Ganoderma stem rot of oil palm (Elaeis guineensis): Mode of infection, epidemiology and biological control

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Award date:
2006

Awarding institution:
University of Bath

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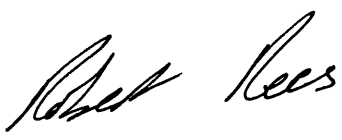
Submitted by Robert William Rees
For the degree of Ph.D.
of the
University of Bath 2006



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Acknowledgements

I would like to express my gratitude to my supervisors Dr Richard Cooper and Dr Julie Flood for their endless help and advice during this project. I'd especially like to thank Richard for his thorough and rapid editing of this manuscript. If this thesis is readable, he is due a large portion of the credit. I owe a huge debt of thanks to Julie for keeping me focused on the important issues relating to the project, for giving me moral support when I needed it and for being so extremely supportive of my career development, thank you very much. I'd also like to acknowledge the great help extended to me by Dr Rob Jackson, who was a great source of advice regarding molecular strategies during the project.

This Ph.D. was conducted in association with LONSUM and many experiments were carried out at Bah Lias research station (BLRS). I would like to pay special thanks to Dr Hugh Foster for supporting this project, for extending me help and for being such a good breakfast/lunch/dinner companion during my visits. I am also extremely grateful to Yonnes Hasan for his invaluable assistance with logistics and practical implementation of field trials; without his help my work at Bah Lias would have been impossible. In addition, Oslan deserves tremendous credit for helping me implement the often complicated trials and for monitoring trials with incredible efficiency, I will always be very grateful. I would also like to thank Dr Steven Nelson for his help, advice and discussions on all things oil palm. I am also very appreciative of the bacon butties, roast beef lunches and the occasional beer with him and Adrine during the visits. Also, *Ganoderma* is a troubling beast to work with and I would like to extend my thanks to Dr Frederick Breton for our mutual *Ganoderma* griping sessions, his good humour and friendship.

Ursula Potter was incredibly helpful for the electron microscopy studies during this project. I would also like to acknowledge the valuable contributions made to this project by Francis Briscoe, Alex Barker, Sarah Norman and Benjamin N. Short. I'd like to express my deep gratitude to Ben. Alongside his excellent work on microscopy of infected oil palm roots, which was invaluable as the basis for further

ultrastructure studies of oil palm infection, I'd like to thank him for being such a good friend; an absolute legend.

During my time at Bath the department was a terrific place to be and numerous people can accept culpability for this; Al Garfunkle, 'The General' aka Ben Short, Tommy Gun, Hilton Macmhaoinigh, Emmsy, Rob Jacko Jackson, Jess Pollitt, Kate Hanrott, Saara Nuutinen, Jane Dickinson, Gaffer Paul Whitley, Dave Clark 5, Pete Millichap, Shifaan, the always serious Chris and Carolyn, Liz Harrison, Iaonnis, Cath Haigh, Mark Hornsey, Amelia Preedy, Ben Heath, Vicky Fleming, Reto Bazzani, Richard Parker, Shazia and all the members of lab 1.52 and write-up room 1.55.

Abbreviations

AFLP	Amplified fragment length polymorphism
AIR	Alcohol insoluble residue (plant cell walls)
ANOVA	Analysis of variance
BCA	Biological control agent
BLRS	Bah Lias research station
BSR	Basal stem rot
CABI	Centre for Agriculture and Biosciences International
CAGE	Cellulose acetate gel electrophoresis
CBH	Cellobiohydrolase
CBL	Column barrier layer
CODIT	Compartmentalisation of decay in trees
CPO	Crude palm oil
CTAB	Cetylmethylammonium bromide
CWDE	Cell wall degrading enzymes
2,4-DAPG	2,4-diacetylphloroglucinol
DMSO	Dimethyl sulphoxide
ED_{50}	Effective Dose 50
EDTA	Ethylenediaminetetraacetic acid
EG	Endo-glucanase
ELISA	Enzyme-linked immunosorbent assay
FD	Frond debris
FFB	Fresh fruit bunch
FP	Fallen palms
GFP	Green fluorescent protein
GM	Gunung Malayo estate
GSM	*Ganoderma* selective medium
IMP	Integrated management practice
ITS	Intergenic transcribed spacer
Lac	Laccase
LiP	Lignin peroxidase
MES	2-N-morpholino ethanesulphonic acid
MLST	Multilocus sequence typing
MnP	Manganese peroxidase
MtDNA	Mitochondrial DNA
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PCA	Phenazine-1-carboxamide
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
PG	Endo-polygalacturonase
PGL	Polygalacturonide lyase
PGPR	Plant growth promoting rhizobacteria
PKO	Palm kernel oil
PNG	Papua New Guinea

PR	Pathogenesis-related proteins
PyAW	Pyridine: acetic acid: water
RAMS	Randomly amplified microsatellites
RAPD	Randomly amplified polymorphic DNA
RBB	Remazol brilliant blue
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RISR	Rhizobacteria-induced systemic resistance
RVU	Relative viscometric units
SAR	Systemic acquired resistance
SBJ	Sonneibijangkar estate
SDW	Sterile distilled water
SEM	Scanning electron microscopy
SIG	Somatic incompatibility group
TAD	Take-all decline
TE	Tris EDTA
TEM	Transmission electron microscopy
TSM	*Trichoderma* selective medium
UPGMA	Unweighted pairgroup method with arithmetic means
USR	Upper stem rot
UV	Ultra violet

Ganoderma Stem Rot of Oil Palm (*Elaeis guineensis*): Mode of Infection, Epidemiology and Biological Control

Stem rot of oil palm, caused by *Ganoderma boninense*, a white-rot basidiomycete, is the main threat to oil palm production in South East Asia. Indonesia and Malaysia produce 80% of the world's palm oil, but losses can reach 50% by the time palms reach 15 years. *G. boninense* can infect both roots and lower stems where it is called basal stem rot (BSR) or upper stems when it is referred to as upper stem rot (USR).

Reproducible root infection was obtained using small, infested wood blocks as inoculum and revealed that intimate association of palm roots with the inoculum source was a prerequisite. This method of infection also showed variation in pathogen aggressiveness and has potential for resistance screening. Under field conditions, temperatures of exposed field soil exceeded 40°C, but *Ganoderma* is inhibited above 35°C; shading markedly enhanced infection and may explain late disease appearance when canopy closure creates shade.

Ultrastructure combined with biochemistry revealed that *G. boninense* rapidly degraded starch, lignin and cellulose and caused extensive breakdown of root cortical cell walls. Subsequent enzyme analysis showed production of all major enzyme groups, which are potential pathogenicity factors.

Randomly amplified microsatellites (RAMS) were used to study epidemiology. High genetic variation of isolates was observed within fallen palms and some BSR infected trees contained several isolates of *Ganoderma*, whereas all isolates within USR infected trees were clonal, thus BSR and USR infections appear distinct. Basidiospores may be important for USR infections and were detected in very high numbers (*ca.* 2,000 - 10,000/m^3) in plantation air samples. Scanning electron microscopy (SEM) revealed that these spores have the potential to germinate on various wounded palm surfaces.

Biocontrol is a possible means of disease control and several strategies were investigated including; isolation of wood-degrading basidiomycetes such as *Pycnoporus* and *Hydnum* spp. for competitive niche exclusion; antagonistic fungi such as *Trichoderma* and *Clonostachys* spp., and bacteria such as actinomycetes (e.g. *Streptomyces* spp.) for active destruction of the pathogen. The most promising isolate was *Trichoderma* SBJ 8, which was shown to be particularly aggressive and its application was shown to reduce infection of seedlings under greenhouse conditions.

Aims

Oil Palm losses suffered by plantation companies as a consequence of the increased incidence of *Ganoderma* stem rot impact both on the amount of oil available for sale and the demand for further land expansion. If understanding of the disease can be increased to facilitate development of successful management strategies, production can be raised and more oil produced from existing plantations. This will help satisfy demand in the rapidly expanding populations of the developing world and at the same time negate the requirement for further expansion into primary rainforest. Thus control of *Ganoderma* infections of oil palm may have both considerable economic and ecological significance. By investigating the epidemiology and pathogenicity of *Ganoderma* stem rot, it is hoped that development of effective control strategies can be achieved.

Infection and Epidemiology of *Ganoderma* Stem Rot

The epidemiology of *Ganoderma* stem rot remains unclear and this study will investigate factors that may contribute to our understanding of disease aetiology. Currently the only established means of infecting oil palm is through root infection; in this study root infection will be utilised to examine various factors that influence infection in the field. This will include the potential of dikaryotic or monokaryotic mycelium to induce infection; determination of the affect of shading and temperature on root infection; size of inoculum necessary for infection and whether wounding significantly increases infection compared to non-wounded roots. Furthermore, random felling of asymptomatic palms from 4-12 yrs and observation of any early disease lesions will be made in order to determine where infections establish on the palm, and at what age infections begin to manifest.

To date no direct infection by basidiospores has been reported. However, the possibility of direct infection of oil palm by basidiospores cannot be discounted in light of the growing problem of upper stem rot (USR). In this study, scanning electron microscopy

will be used to assess the ability of basidiospores to germinate on various oil-palm wound surfaces; namely fronds, fruit stalks and trunk tissue, both on the surface and within xylem vessels. Currently there are no accurate estimations of atmospheric basidiospore numbers and inflated levels of basidiospores may increase the incidence of USR. In this study a centrifugal air sampler (Biotester®) will be used to provide an accurate estimation of basidiospore numbers within different aged plots in a plantation in Sumatra.

Molecular studies to date have indicated that *Ganoderma* populations within oil palm plantations are genetically diverse with genetically distinct individuals often present on adjacent infected trees. In this study, the molecular profile of individual infections of basal stem rot (BSR) and USR will be investigated using randomly amplified microsatellites (RAMS) to determine if more than one isolate can occur within a single palm and whether both BSR and USR infections show the same profile. Fallen palms and adjacent BSR infected trees will also be investigated to see whether isolates contain genetically identical isolates..

***Ganoderma* Pathogenicity and Wood Decay**

Although *G. boninense* has been widely accepted as the pathogenic agent for BSR and USR in oil palm, there have been no detailed ultrastructural studies of the plant-pathogen-interaction during BSR infection of oil palm. The pathogenicity of many other plant pathogenic fungi can often be apportioned to enzyme production but this information is almost completely unknown for *G. boninense*. Ultrastructure studies will be conducted using light and electron microscopy of infected roots and basal stem tissue of 18-month old juvenile palms. This will provide the first evidence of the process of infection by *G. boninense* and how the pathogen degrades oil palm tissue and progresses through the host.

Enzymatic studies will be performed to compile a profile of the spectrum of enzymes produced by the pathogen when degrading oil-palm tissue. Knowledge of the enzyme array produced by *G. boninense* will facilitate more accurate interpretation of ultrastructure images from infection and may reveal some candidate pathogenicity factors

for future genomics studies. Moreover, knowledge of the enzymes produced by *G. boninense* will provide information that will be fundamental for the development of tailored control strategies.

Investigating Biological Control of *Ganoderma* Stem Rot of Oil Palm

Current disease control strategies for BSR are ineffective and cultivation of the crop using extensive monoculture serves to exacerbate the problem by providing ideal growth conditions for the pathogen. One possible method of combating the problem is to identify key pathogen reservoirs and attempt to release competitive/antagonistic organisms to challenge *Ganoderma*'s dominance of these. By utilising knowledge of the pathogen built up by studies described above, a strategy of isolating both competitive wood degrading and antagonistic organisms will be made, predominantly from decaying oil palm tissue. The ideal strategy would be to formulate a mixed inoculum of organisms antagonistic to *Ganoderma* plus competitive wood-degrading organisms for release onto felled/toppled oil palm within plantations. A combined inoculum would thus have the ability to actively inhibit *Ganoderma* colonisation of the material and initiate degradation of the oil palm by non-pathogenic organisms, thereby starving the pathogen of food-source and reducing the inoculum threat posed by decaying palm material.

In this study all isolated organisms will be tested *in vitro* for antagonistic activity against *G. boninense* and against one-another using dual plate assays. The most aggressive *Ganoderma*-antagonistic fungi and bacteria will then be selected for further assessment of efficacy against *Ganoderma*. This will be achieved by determining ability to inhibit *Ganoderma* colonisation of oil palm wood *in vitro* and ability to reduce infection compared to controls under greenhouse conditions. Wood-degrading organisms will be investigated for their ability to degrade oil palm wood and their polymer utilisation in order to select organisms that utilise components shown to be critical for growth and development of *G. boninense*. Initial degradation studies will be conducted in the laboratory and promising isolates subsequently tested in field trials in Sumatra. All promising biocontrol organisms will also be monitored for any signs of pathogenicity towards oil palm to ensure that these organisms are saprophytic.

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2 INFECTION AND EPIDEMIOLOGY OF *GANODERMA* STEM ROT 31

1 General Introduction

1.1 Oil Palm

The Oil palm (*Elaeis guineensis*) is a monocotyledon in the Arecaceae (formerly known as Palmae) family, within the subfamily Cocosoidae (97). There are three species of *Elaeis*, *E. guineensis* (commercial red oil palm) from Africa, *E. oleifera* (American oil palm) from South America and *E. odora,* which grows in the Amazon rainforest, South America. Naturally growing oil palms are usually found near rivers with plentiful, but not excessive moisture, where light penetrates and where there is less competition from other forest flora. *Elaeis guineensis* originates from West and Central Africa but has now spread throughout the equatorial tropics including: Malaysia, Indonesia, India, Philippines, Papua New Guinea, the Solomon Islands, Ghana, Angola, Cameroon, and Honduras (14, 232). More than 95% of the world's oil palm is grown within the range 10° north and south of the equator. Climatic factors such as severe drought, surplus rainfall, wind gusts, sunshine and diurnal temperature range all influence oil palm crop performance (97).

The value of oil palm as an oil-yielding crop was first realised in the mid 1800s when collapse of the slave trade induced natives of Guinea coast to begin processing and selling oil for export to replace the trade in slaves. Exports of palm oil from Africa steadily rose between the 1860s and the early 20th century to meet the demands of industrial Europe. In 1911 87,000 tonnes of palm oil valued at £2 million, were exported from British Colonial Territories in Africa in a single year. Efforts to introduce more efficient production methods were largely unsuccessful, and this coupled with civil unrest and conflict has led to a steady decline in production from most African nations during the 20th century. Today only the Democratic Republic of Congo and Ivory Coast of the African nations produce significant amounts of oil palm, consequently exports from this region make up only a small minority of world percentages.

Four palms of the Deli type were brought to Indonesia in 1848; two from West Africa and two from Holland, and were planted in the Botanic gardens in Bentenzorg. Bogor. Java. Their progeny were distributed from 1853 onwards and formed the basis of the oil palm industry in Indonesia (previously the Dutch East Indies). Foundation of the industry is attributed to M. A. Hallet, a Belgian with some knowledge of the oil palm in Africa, who in 1911 planted Deli origin palms in plantings on Sungei Liput, Atjeh and Pulu Radja. The first commercial planting in Malaysia began in 1917 at Tennamaran estates in Kuala Selangor. From this beginning the cultivation of oil palm in Malaysia grew rapidly with 16 ha planted in 1920, 231 ha in 1922 and 409 ha in 1923. Malaysian oil palm cultivation continues to expand and covered over 2 million ha by 1988 (97).

1.2 Oil Palm Industry and Economic Importance

Oil palm can grow to 20-30 metres and typically has a productive life of 25 years within plantations. During prime production (11-25 years) yields can be as much as 6-8 tonnes oil ha^{-1} $year^{-1}$ (48). Between 1989 and 1991 11.36 million tonnes of palm oil was produced accounting for 18.6% of the world's total vegetable oil production, second only to soybean oil (16.130 million tonnes, 20.2% of world oil production) (63). Oil palm holds advantage over all other oil producing crops since a single tree can be harvested several times each year allowing greater oil production than alternative oil producing crops and is a crucial source of cheap oil for developing nations such as China and India. Asia is the main producer and oil palm has attained prominence as a plantation crop in many of the Pacific-rim nations. Cultivation in Asia over the last few decades has grown enormously from just under 200,000 ha of oil palm production in the 1960s increasing to 6.5 million ha in 2005, accounting for almost 90% of world production (59). This represents a large proportion of Malaysian agricultural production and 10% of Malaysian land mass was covered by the crop by 2003 accounting for 56% of agricultural land area (101). Malaysia is the world's greatest exporter of palm and its value to the Malaysian economy is second only to crude oil.

From oil palm two grades of oil can be produced: crude palm oil (CPO) comes from grinding fruit pulp (Fig. 1) to release standard quality oil used in biscuits, margarine and cooking oils, and palm kernel oil (PKO) which is obtained from grinding the nut to release high quality oil used in pharmaceuticals. In 1997, Indonesia alone produced 5,904,175 t of CPO with a value of US $2,952,087,500 and 1,189,603 t of PKO with a value of US $832,722,100, a combined total value of $3,784,809,600 (52).

FIG. 1. Freshly harvested fruit bunches of the tenera variety commonly grown in South East Asia.

Given the scale of oil palm production and the high commercial value of its vegetable oil, pests and diseases that seriously impair yield could have far ranging implications. As with other plantation crops, there are a great many diseases and pests of oil palms and these are covered in detail by Corley and Tinker, 2003 (48). However in South East Asia the greatest threat to sustainability is from basal stem rot (BSR) and upper stem rot (USR), diseases caused by *Ganoderma boninense*.

1.3 *Ganoderma* Taxonomy

Ganoderma Karst, of the family Ganodermataceae, order Polyporales, was established by Finnish mycologist Peter Adolf Karsten in 1881 for the laccate and stipitate white rot fungus *Polyporus lucidus*. It was later amended by Patouillard (1889) to include all polypores having double walled basidiospores and listed 48 species worldwide in his treatment of the genus (147). Later Murrill published a synopsis in 1908 of species occurring in North America describing several new temperate species including *G. tsugae*, *G. sessile*, *G. zonatum*, *G. sulcatum* and *G. oregonense* amongst others (4). *Ganoderma* spp. have now been shown to have a worldwide distribution growing on numerous coniferous, deciduous, and palmaceous hosts (142). Taxonomic studies have traditionally used characters such as host specificity, geographical distribution and macromorphology of basidiophores (including context colour, shape of pileus margin and stipitate/sessile) as a basis for speciation. A recent study focused on spore size, context thickness and colour, pores, cuticle thickness, tubes and the angle and diameter of cap margin (124). Many of these features are polymorphic within the same species and this has led to taxonomic confusion, approximately 30% of names in *Ganoderma* have been proposed as synonymous (211). Ryvarden (188) suggests that caution must be taken when using traditional morphological characters to reach taxonomical conclusions. The pileus for example can change size and shape with age and is a dubious choice for morphological study. Its colour may also change, such as from yellow to deep bay or almost black with age, as in *Ganoderma lucidum*. Context colour is also variable, especially in dried specimen material and differences between context colours of Portuguese and Finnish specimens of *G. lucidum* have been reported (215).

Stipe attachment and size and shape of basidiospores are more consistent factors for taxonomic study, however Ryvarden (188) suggests a study of several collections must be made even for these features before reaching conclusions. In the past two decades attempts have been made to bring order to the family using these and other more reliable taxonomic characters for morphological study. Light and electron microscopy have revealed that several characters of basidiospore morphology are useful for between species comparison but remain consistent within species. For example, variation exists in size and shape between species but variation within

species was found to be low. Adaskaveg & Gilbertson (3) found that basidiospore cell walls are complex and composed of several layers connected by inter-wall pillars, which differed in shape and distribution. Studies of several species of *Ganoderma* from North America was used to provide a culture profile including: minimum/maximum temperature range, optimum temperature for hyphal extension *in vitro* and oxidase reactions on tannic acid agar plates, intended for use in conjunction with other consistent morphological taxonomic markers (5). Interfertility tests using monokaryons derived from individual basidiospores showed that all *G. lucidum* isolates were incompatible with *G. tsugae*, suggesting this might also be a useful tool for speciation (4).

More recently molecular characterisation by internal transcribed spacers (ITS) and divergent domain (D2) of 25S rDNA has revealed the extent of parallelism or convergence in the evolution of morphological features. Parsimony analysis revealed that three isolates of *G. tsugae* and three isolates of *G. resinaceum* did not cluster together (146), suggesting these may not be the same species. In the *G. lucidum* species complex, strains showing less than 1.5% sequence differences are putatively conspecific. This work showed that there was little correlation between rDNA gene phylogeny and morphology in the *G. lucidum* complex and underlines its phenotypic plasticity, which has been evidenced previously where geographically separated *G. lucidum* isolates were reported to have different coloured contexts (189). However, clades produced by molecular characterisation are supported by characteristics of mycelia in culture, geographic origin, host relationships and mating data. Smith & Sivasithampathan (209) also sequenced ITS regions to study *Ganoderma* species from Australia and produced well supported groups, identifying further anomalies with current nomenclature. Isozyme analysis by this group, using cellulose acetate gel electrophoresis (CAGE) and polyacrylamide gel electrophoresis (PAGE) supported their groupings produced by ITS sequencing (210). Analysis of pectinase isozymes were also used by Miller *et al* (144) to characterise *Ganoderma* strains from South East Asia and resulting groupings suggested that species of *Ganoderma* may be separated according to their host specificity as 99% of a major cluster group were obtained from oil palm. In addition, seven of the remaining isolates in this group were isolated from coconut palms and isolates found in coconut are also often found in oil palm. Other molecular tools such as RAPDs have also been studied for

phylogeny of species in the *Ganoderma lucidum* complex, but groupings were poorly supported by bootstrapping (106). Restriction fragment length polymorphism (RFLP) of ITS regions for *Ganoderma* populations on coconut and oil palm has also been conducted in Malaysia (87). This technique revealed that almost all of these isolates were inseparable using RFLP and are thus closely related; however this technique is not as powerful as direct sequencing and cannot differentiate between closely related species. Investigators agree that use of molecular biology is a powerful phylogenetic tool but must be carefully used in conjunction with interfertility testing, host range data and cultural information for speciation.

1.4 *G. boninense* Morphology

Although polymorphism in *Ganoderma* is problematic for taxonomists, knowledge of general morphological features is useful when working with *G. boninense*. A useful morphological guide for the *Ganodermataceae* may be found in 'Genera of the Polypores' by Ryvarden (189). Two kinds of *Ganoderma* basidiophores have been distinguished: those with shiny (laccate), yellowish or reddish-brown to black pilear surface, and those with a dull (non-laccate), grey-brown to black pilear surface (145). Consistent with the findings of Ryvarden (188), Pilotti (171) considers few features of *G. boninense* basidiophores to be reliable characters for separation at the species level; the most reliable being surface texture, colour and context colour. However, basidiospore size and shape morphology are the most dependable characters (171).

Ganoderma boninense basidiophores have a smooth red-brown to black laccate pileus depending on age, are usually stipitate or dimidiate and have fine concentrically grooved surfaces (175) (Fig. 2). The pore surface is typically creamy-white with production of basidiospores 9-11 μm in length. Basidiospores are melanised, have an apical germ pore, which varies in appearance and most have a large vacuole. *G. boninense* is typical of the genus with ovoid echinulate basidiospores that are truncated at the apex.

FIG. 2. Basidiophores growing on a basal stem rot (BSR) infected oil palm. **5a** Mature basidiophores with pilear surface and white pore surface. **5b** Shiny laccate, red-brown coloured pilear surface of fresh fruiting body with a prominent white lip at the pilear margin.

1.5 *Ganoderma* Stem Rot of Oil Palm

Root and stem rots caused by *Ganoderma boninense* were first described in 1915 in the Republic of Congo, West Africa as a disease of senescing palms (246). The disease was first identified in peninsular Malaysia by Thomson in 1931 (227) who originally identified the causal agent as *Ganoderma lucidum*. As in Africa the disease was typically only found in older palms (>20 years) and was described as not economically important. Later, Steyaert (1967) identified six *Ganoderma* species isolated from oil palm fields, *G. boninense, G. miniatocinctum, G. chalceum, G. tornatum, G. zonatum* and *G. xylonoides*. As with the genus *Ganoderma* in general, considerable uncertainty surrounding identity of the causal agent of BSR has led to many authors simply referring to the disease as '*Ganoderma* rot of palms' (48). However, recent studies have indicated that *Ganoderma boninense* is the primary cause of the disease in South East Asia and other identified species are synonyms or due to secondary invasion of existing infections (14, 171, 172). This project serves to focus on *Ganoderma boninense* as the causal agent of oil palm stem rot diseases BSR and upper stem rot (USR) in Indonesia, Malaysia and Papua New Guinea.

Symptoms of *G. boninense* infection can vary depending on age of the plant and climatic conditions. Foliar symptoms usually only occur after over 50% of the radius of the basal region of the palm has been decayed. The symptoms first manifest as chlorosis of palm leaves on one side of the palm, or 'one sided mottling' with newly opened fronds (leaves) being smaller and paler than normal (207). Later leaves do not open at all and lead to multiple unopened fronds, termed 'spears' due to their pointed morphology. As infection progresses the palm takes on an overall pale appearance and affected leaves eventually die with leaves drooping and fracturing at the base (Fig. 3).

FIG. 3. Infected and healthy mature oil palms. **a** shows BSR infected tree with drooping fronds and one-sided chlorosis, typical of advanced infection often accompanied by production of *Ganoderma* basidiophores at the base of the palm. **b** shows healthy canopy of mature uninfected oil palm. Fronds are uniformly green and arrayed to maximise light capture.

Once infected, young palms normally die within 6-24 months after first appearance of symptoms, whereas mature palms can take 2-3 years or more to die. These symptoms are not diagnostic of *Ganoderma* stem rot as similar symptoms can also be seen during water stress (232). Indeed, a palm may be heavily infected with almost half of the stem degraded yet have no foliar symptoms or basidiophores. Therefore there is not always a correlation between foliar symptoms, formation of basidiophores and degree of infection, making it difficult to diagnose early infections. In Malaysia it was reported that 13-22% of palms showing no foliar symptoms or yield loss in 22-year old palms were actually infected with *Ganoderma* on inspection of bole transverse sections (48). Hasan & Turner (99) also noted the prevalence of sub-clinical infections during experiments using bait seedlings where comparisons were made between infection rates of seedlings planted adjacent to BSR and apparently healthy trees. In one instance, 40% of seedlings planted adjacent to an apparently healthy mature palm (two palms distant from the nearest BSR infected neighbour) became infected within 2 years, with later manifestation of symptoms on the standing mature palm indicating that the palm may have had a sub-clinical infection. Thus

sub-clinical infections are difficult to identify and may go unnoticed for long periods; the only truly diagnostic feature of the disease remains the presence of basidiophores either at the base, or higher up the stem in the case of USR.

Presence of basidiophores on the stem surface indicates extensive internal rotting; analysis of cross-sections through rotting areas shows soft discoloured areas of rot where extensive colonisation of the tissue and degradation of cell wall polymers has occurred (Fig. 4). This is bordered by distinct dark brown margins which marks the advancing edge of invasion and is termed the 'reaction zone' (232). Pearce (164) describes reaction zones as 'a static barrier that serves to restrict or slow fungal ingress through a combination of factors including deposition of phytoalexin-like compounds such as phenolics and polyphenolics as well as microenvironmental factors' (Reaction zones are described in greater detail in chapter 3). The dark colouration of reaction zones may in part be due to fungal stress responses inducing formation of pigmented, thick cell walled hyphae. In saprophytic decay wood, margins of colonisation are often delimited by 'pseudo-sclerotial plates' and have been shown to be produced by other *Ganoderma* species including *G. australe*, responsible for white-rot of wood tissue in Chile (9) and similar formations have been formed by *G. boninense* on oil palm blocks during this study (Fig. 5). Pseudo-sclerotial plates have been suggested to play a role in compartmentalising decay tissue, protecting the nutrient source and the fungus itself, from desiccation and competitors in saprophytic wood degradation. Immediately in front of this reaction zone, stem tissue is a pale yellow colour and may be caused by some form of host defence responses. USR rot patterns have a more organised morphology with sequential areas of rot that spread in waves from the point of initial infection (98) (Fig. 4b). BSR decay patterns are more disorganised with a greater occurrence of highly defined pseudo-sclerotial plates (Fig. 4a). This suggests there may be multiple points of infection and more inter-specific and intra-specific competition in the basal lesion. It is this extensive decay of woody palm tissues that result in foliar symptoms, presumably due to a disrupted vascular system, and ultimately palm death and toppling due to compromised structural integrity.

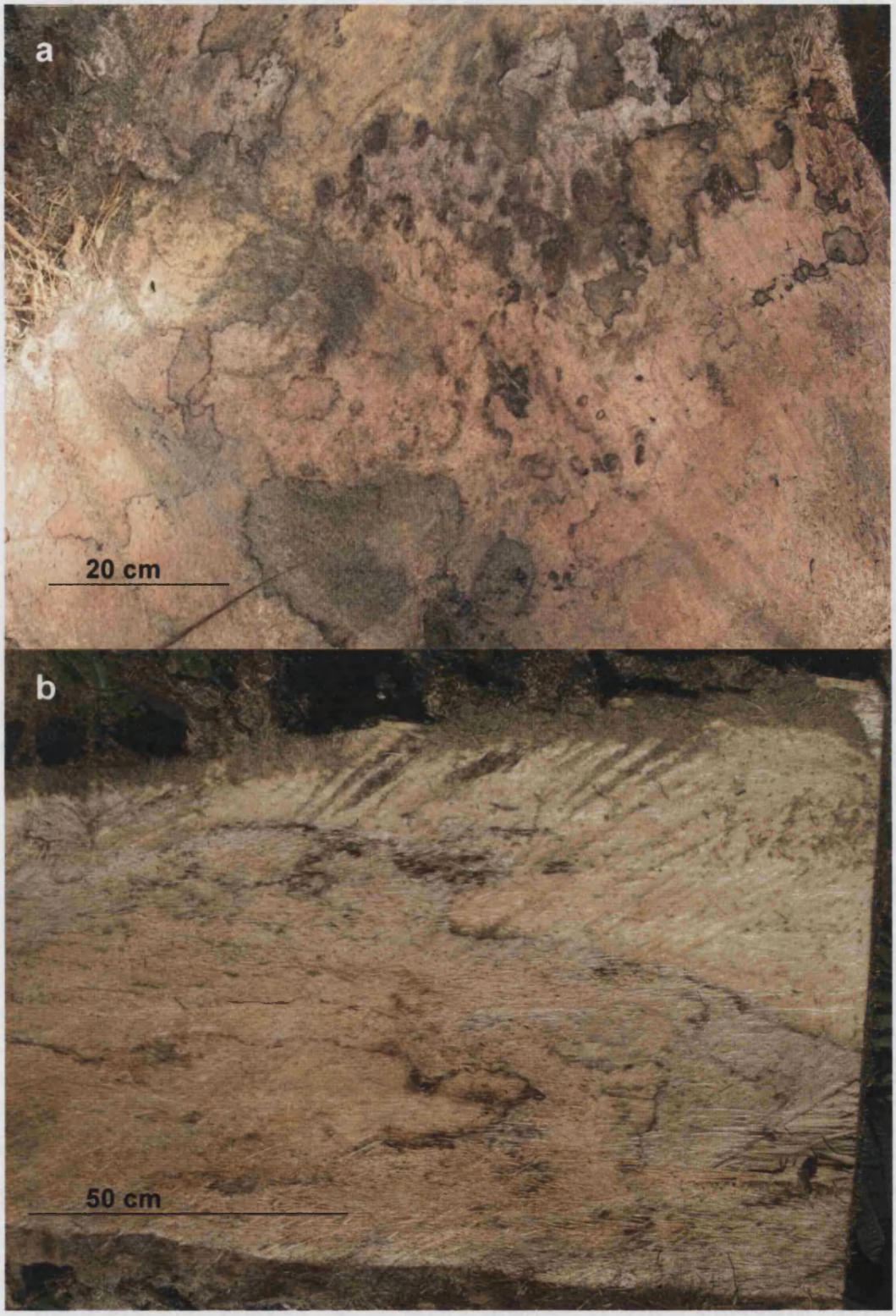

FIG. 4. Decay patterns of BSR and USR disease lesions. **a** Transverse section through the base of a BSR infected palm shows multiple lesions and development of demarcation lines, compartmentalising decay areas. **b** shows the more organised pattern of decay typical of USR infections with ever-expanding layers of an organised decay front, suggestive of less competition than in BSR infections.

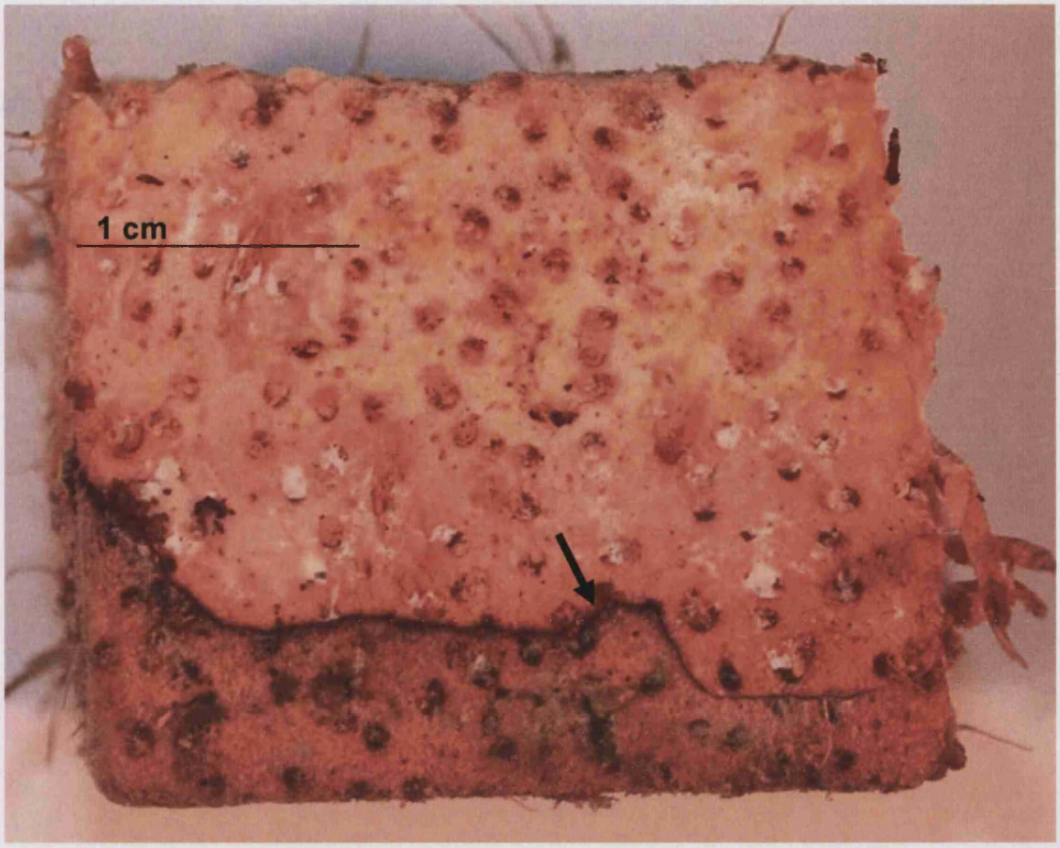

FIG. 5. Oil palm wood block partially decayed by *Ganoderma*. Upper bleached section of wood showing white-rot decay due to lignin peroxidase and other cell wall degrading enzymes from *G. boninense*. Distinct demarcation line (arrow), is probably a pseudo-sclerotial plate formed by thick walled hyphae, separating the decayed area from non-decayed tissue

1.6 *Ganoderma* Stem Rot, an Increasing Problem

Since the 1960s, oil palm has assumed prominence as a plantation crop in South East Asia. During this time *Ganoderma* stem rot incidence has increased, frequently infecting younger palms (10-15 years), leading to suggestions that monoculture of oil palm has had a role in rising disease incidence (232). Most severe losses from *Ganoderma* stem rot occur in Indonesia and Malaysia with less severe losses being recorded in the Democratic Republic of Congo, Ghana, Nigeria, Cameroon, Angola, Zimbabwe, Tanzania, Papua New Guinea and the Solomon Islands. The disease has now also been described in Thailand where cultivation of the crop has occurred more recently with 2 out of 2000 20-year old oil palms showed typical symptoms (128). Increased incidence has been reported in Malaysia, where Singh observed infected

palms as young as 12-24 months after planting with increased rates in 4-5 year old palms in coastal alluvial soils (207). Similarly, Lim reported an average of 50% yield losses from 80% of 13 year old plantings in Malaysian coastal areas (130). A recent survey by Rao *et al* (180) further demonstrates the increasing problem of *Ganoderma* stem rot of oil palm. They reported typical disease levels of 30% by the time palms were 13 years of age in inland and peat soils of Malaysia. Whereas in the coastal areas incidence of the disease has been known for many years, these inland areas have had typically low or negligible incidence of BSR or USR until recently, and losses reaching levels comparable to those on the coast increases concern for sustainability.

In Indonesia, plantations encounter similar problems. At Bah Lias in north Sumatra, losses of up to 25% occurred within 7 years in one plantation block with general levels of infection across the plantation impacting on yield after 14 years. Generally in North Sumatra, by the time of replanting (25 years) 40-50% of palms are typically lost with the majority of standing palms showing symptoms of infection. Losses begin to have a financial effect once losses reach more than 10% of the stand (99). On average there is a fresh fruit bunch (FFB) yield decline of 0.16t/ha for every palm lost and when the stand had declined by 50% the FFB yield reduction was 35% (Hugh Foster pers. com). Similarly high yield losses have been encountered in other plantations where average yields for an uninfected plot was 388 kg/ha compared with 291.9 kg/ha in a plot with 35% BSR-induced fallen palms (230).

1.7 Factors Associated with BSR

1.7.1 Palm Age

Though BSR was first described as a disease of senescing palms, Turner (232) postulated the age at which a palm becomes infected would depend on a number of factors: i) the rate of colonisation of the tissues of the previous stand: ii) proximity of the colonised tissues to the oil palm iii) time taken for roots to make contact and become infected: iv) growth along the root and its establishment within bole tissues. One study in Malaysia determined that an isolate of *Ganoderma* colonised roots *in vitro* at a rate of only 1 cm/month (108). Even at infection rates greater than this it is

not difficult to imagine how it could take many years between initial challenge and disease manifestation. The rate and extent of growth of palm roots is considerable and Miller *et al* (143) described palm roots extending across up to four planting rows and Darmono (52) considered mature oil palm roots to cover more than 16 m^3. Nevertheless, Hasan and Turner (99) reported that when a suitable disease source is present, oil palm may be attacked soon after planting. It is therefore tenable that palm infection is less affected by palm age than by presence of inoculum and length of time for disease to establish.

1.7.2 Crop History

It appears clear that crop history is an important factor for palm and yield losses in future generations. Turner reported that planting after coconut and oil palm resulted in greater losses in the subsequent generations than were observed when planting after forest or rubber. In two 15 year-old fields planted after rubber, the disease levels were 2 and 4 % respectively, compared with 39 and 35% in fields planted after coconut (232). The highest rate was observed in a field where coconut trees were buried to prevent infestation by the rhinoceros beetle (*Oryctes rhinoceros*) with infection rates of 81%. Turner considered oil palm tissue to have a lower inoculum potential than coconut, based on surveys showing infection rates of 24-28% in 15-year-old palms after oil palm and 6-81% after coconut.

More recent data shows comparable disease levels in plantings after both oil palm and coconut with infection rates as high as 67% after oil palm (48), whilst rates subsequent to forest or rubber remained low up until replant. Increased rates of disease subsequent to coconut might be expected since *Ganoderma* spp., notably *G. lucidum* (which is often a misnomer for *G. boninense* in the tropics), *G applanatum and G. colossum* are associated with an analogous basal stem rot disease of coconut (153, 196) and that *G. boninense* grows readily on dead coconut tissue (172). Recent increases in plantation infection levels have been reported to be due to a build-up of inoculum and that this build-up is additive from generation to generation (232). This is difficult to prove however since the 25-year time intervals between generations makes reliable comparisons highly problematic since estate management practices are likely to have changed during this time and these would also have an influence on

disease incidence. Also, if different oil palm generations are not the same genotype then possible genetic resistance could distort disease levels between generations (59). Nevertheless increased incidence in plantings subsequent to oil palm and coconut is an accepted trend in the oil palm industry.

1.7.3 Soil Type and Nutrient Status

In Malaysia coastal areas have frequently reported high incidence of BSR. In these areas soil is mainly clay, silty clay or clay loam with poor drainage and with high water retention capacity (14). However, recent years have seen a rise in BSR on inland peat and lateritic soil plantations. High water content is also implicated in these plantations and in one study the worst affected areas were seen to follow former watercourses (180). Coastal regions were the first areas to be turned over to oil palm production and many of these estates are now second or third generation. Inland regions have been converted to oil palm cultivation more recently and previous low incidence may have been due to the predominance of first generation plantings.

Soil nutrition may also influence disease but the situation is unclear. Recent studies indicate that use of calcium nitrate as a fertiliser may reduce incidence of BSR. Although this is stimulatory to growth of *G. boninense*, addition one month before inoculation of seedlings reduces infection by the fungus (198). Action may be due to enhanced health of the plant or increased concentrations of saprophytic fungi and bacteria (9). Numerous other studies have been conducted to investigate the affect of fertilisers and major elements on disease, which are summarised by Ariffin *et al* (14). There appears to be no clear link between disease incidence and any particular fertiliser or element, and in some instances researchers have reached contradictory conclusions. Due to inconclusive studies neither soil type or nutrient status has been shown to be critical in determining levels of infection in the field.

1.8 Replanting Strategies

Land preparation at the time of replant has been regarded as important for the exclusion of *Ganoderma* in the subsequent crop and is particularly important in plantings after coconut and oil palm. There is *ca.* 85t/ha dry weight of palm material

to be disposed of at the end of the productive life of a crop and before the ban on burning was introduced in South East Asia, the most common method of clearing old oil palms for replanting was the "chip and burn" method (160). Under the zero burn policy there are three main land preparation strategies; namely under-planting, clean-clearing and windrowing. These have been widely practised throughout the region and work on the assumption that infection occurs through root-to-root or root to inoculum contact. This assumption is supported by extremely high levels of *Ganoderma* infection in oil palm planting subsequent to burying the previous generation of coconut palms (232), and 93% incidence of BSR infection in palm seedlings planted around the base of an infected palm (14). Therefore rigorous land preparation before planting, despite the inherent high costs involved is deemed critical within the oil palm industry. Recently due to concerns over the proliferation of *Oryctes rhinoceros* and rats in plantations where windrowing is practiced, a method involving the pulverisation of oil palms has also been introduced in Malaysia (160).

1.8.1 Underplanting

Smallholders commonly use underplanting, as old palms continue to provide a return whilst juveniles are in the development phase. The process involves underplanting mature palms with young oil palm, followed by poisoning and felling of the old stand, which is stacked and left to degrade in the inter-row, usually two palm rows to one windrow (2:1). However, subsequent losses due to BSR are high and the practice has long been discouraged within plantations (232). Losses in the subsequent generation may also be incurred by the rhinoceros beetle (*Oryctes rhinoceros*), which can use the remaining tissues of the old stand as a breeding ground.

1.8.2 Clean-clearing

Clean-clearing is regarded as the most desirable sanitation measure despite the high costs involved. Palms are toppled when they reach 25 years and remaining bole fragments are removed from the soil by digging pits 1.5 m square and 60 cm deep and placed on the surface for removal, based on recommendations from Hasan and Turner (99). However high cost of removal means that common practice is to shed all palm fragments. These are then stacked in inter-rows (2:1) and covered with a legume crop to facilitate decay and to deter colonisation of the palm debris by *Oryctes*. Although clean-clearing generally results in lower disease incidence in replanted oil palm, it has

been reported that planting of legume cover crops may encourage colonisation by *Ganoderma* spp.

1.8.3 Windrowing

Windrowing (Fig. 6) is a replanting technique based on the same principles as clean-clearing. It involves removal of all primary sources of inoculum from the soil by digging out the bole as explained above. Palms are then planted in the inter-rows at a recommended distance of at least 3 metres from windrowed material (78). These tissues, together with felled trunks, are stacked unshredded along the line of previous planting and allowed to degrade by natural processes. Legume crops are again planted to reduce *Oryctes* infestation, however considerable colonisation of trunk tissues is inevitable (Fig. 6.). Also presence of *Ganoderma* basidiophores in these windrows is extensive and may be possible infection foci for future generations. Turner and Hasan reported that *Ganoderma* colonised trunks of oil palm remain infectious for at least 2 years (99). Despite this, losses of trees in subsequent generations are comparable to the clean-clearing technique and only begin to affect yield after about 14 years. Windrowing is the practice commonly employed by LONSUM plantations and other plantation companies throughout North Sumatra, Indonesia.

1.8.4 Palm Pulverisation

Oryctes rhinoceros is now regarded as the most serious pest in immature and young oil palms in Malaysia, inducing losses of over 40% in the first year of harvesting (160). Oil palm trunks left to degrade in windrows take more than two years to degrade, however it takes just 3.8 to 8.6 months from egg laying to emergence of the full adult *Oryctes* beetle (22), making windrows perfect breeding grounds for *Oryctes* (Fig. 6). To address this several high power pulverising/shredding machines have been developed that can dig up the root mass and pulverise wood tissue into fine fragments, which can be then scattered throughout the plantation. It was reported that 50 kg (dry weight) of the pulverised palm biomass is able to supply the entire N, P, K and Mg requirement of a palm in its first year of growth (160). However, the financial cost for the purchase, running and maintenance of pulverisors/shredders may prove prohibitive in many instances. Additionally, variation was reported in the

quantity of palms that can be processed each day and this is another consideration for large estates at replant. Authors suggest that one of the advantages of this method, alongside reduction of rat and *Oryctes* problems, is it may be possible to reduce the fallow period following the previous crop as *ca.* 80% of dry matter was reported to be degraded after 56 weeks (160). However it is still unknown how small an inoculum is required to establish *G. boninense* infections or how long *Ganoderma* resting structures can survive in the soil, therefore care must be taken before deciding to shorten fallow periods.

FIG. 6. Felled oil palms stacked in rows called "windrows" and larvae of *Oryctes rhinoceros* that develop within decaying palm wood. **a.** Windrowed oil palms left to degrade in the field with young palms planted in the inter-rows, arrows. **b**. Larvae from *Oryctes rhinoceros*, which burrow into the dead palm tissue and uses these as a breeding ground. Larvae feed on the decaying wood and adult *Oryctes* cause damage to seedlings by eating the immature leaves.

1.9 Early Detection of *Ganoderma* Stem Rot

Diagnosis of *G. boninense* infections of oil palm remains difficult at the subclinical level and by the time foliar symptoms or basidiophores develop much of the palm stem has often been decayed. Early detection of the fungus would enable decisions to be made on treatment of infected trees or preventative control measures to limit spread of the disease. Development of diagnostic methods to detect pathogenic *G. boninense* isolates could also be useful for monitoring pathogenic populations in the environment and determining potential reservoirs. These requirements were noted by Samiyappan *et al* (192) who described several possible methodologies that could be used for early detection of infection, including nucleic acid hybridisation, polymerase chain reaction (PCR) and use of polyclonal or monoclonal antibodies.

Polyclonal antibodies have been investigated for their potential to detect presence of *G. boninense* isolates using enzyme linked immunosorbent assay (ELISA) (233, 234). The ELISA method was investigated under laboratory conditions using pure cultures of several fungi (*Trichoderma*, *Penicillium* and *Aspergillus*) and showed low cross-reactivity. However, although cross-reactivity was low, for practical purposes a positive reaction, however low, may lead to incorrect detection. For this reason the authors suggest use of the ELISA assay for rapid initial screening followed by more reliable use of rDNA PCR using species specific primers to confirm positive findings. Species specific primers IT1, IT2 and IT3 were developed from variable ITS regions after first amplifying using universal fungal primers ITS1 and ITS4 (233, 235). The products of these reactions were then analysed using restriction fragment length polymorphism (RFLP) for practical use, since few plantations in Malaysia and Indonesia are likely to have ready access to sequencers. A similar PCR method was developed by Bridge *et al*, where a primer combination ITS3/GanET was shown to produce a 321 bp product that was specific to pathogenic *Ganoderma* isolates (32, 33). This could allow determination of pathogenic isolates based on product size and does not require culturing of the fungus, as it can be used directly from oil palm tissue. However, the above methods all require a limited amount of palm wounding to access potentially diseased tissue, which could encourage infection of healthy trees. In addition, each of the methods requires some specialised equipment and scientific knowledge, which may preclude their use for many plantation companies.

1.10 Control of *Ganoderma* Stem Rot

Effective control strategies for BSR and USR of oil palm has so far proved elusive; the best means of combating infection remains the maintenance of sanitary conditions at replant though rigorous land preparation. To minimise losses from *Ganoderma* infections both long and short-term strategies for disease control are necessary.

1.10.1 Cultural Control

Cultural control practices such as trenching have been recommended since the early twentieth century. Trenching has been extensively used in the oil palm industry and is designed to separate the root system of infected palms as a quarantine measure to protect neighbouring healthy palms; this assumes spread of the pathogen occurs through the root system. The method has been largely unsuccessful in preventing proliferation of the disease perhaps because of insufficient depth of the trenches or failure to maintain trenches once in place. Furthermore, digging trenches not only severs roots of infected palms but also roots from healthy palms. Should spores play a role in direct infection of oil palms these cut roots would represent another wounded surface for basidiospore germination and development of disease. Also, dissemination of infection by basidiospores rather than by root contact would render the digging of trenches inappropriate.

Mounding of soil around infected palms has also been utilised as a method of extending the productive life of infected palms. This stimulates renewed root development from stem tissue above the infected region and provides added stability, which compensates for reduced stem structural integrity due to decay by *G. boninense*. However, this treatment simply extends the life of the palm but does nothing to prevent the course of infection or transmission of the fungus (230).

1.10.2 Curative Surgery

Manual excision of infected tissues from rot lesions in outer stem tissues of infected palms was a widely adopted treatment strategy in the 1970s. After excision, the cut surface was treated with a protective chemical (e.g. coal tar or a mixture of coal tar and thiram) (14). However, necessity for regular inspection of trees to identify BSR and mixed results in treated palms led to a decline in the practice. Age of palms is a

factor when considering this method; when basidiophores are observed in young palms the extent of decay is often too far advanced for surgery, whilst palms over 20 years are very tall so excision of even small lesions can result in toppling (232).

1.10.3 Chemical Control

Effective use of chemical control for treatment of BSR infected palms is problematic since both symptomatic and subclinical palms may harbour advanced infections (200). Contamination of fungicides and fumigants in palm oil may also preclude widespread use within the industry. Fungicide resistance of the fungus has not been a concern and numerous fungicides have been shown to be inhibitory to *Ganoderma in vitro*: Hexaconazole, cyproconazole and triadimenol gave ED_{50} (amount required to produce an effect in 50% of subjects) values of 0.03, 0.043, and 0.06 ppm active ingredient respectively (141). Effective treatment in the field has proven more difficult and lesions are often very large, making delivery difficult. For example, lesions are predominantly at the base and high-pressure injection often results in passage of the chemical into the soil. However, application of the systemic fungicide triadimenol was shown to prolong the life of infected palms (14). So far chemical control of BSR has been limited because of inconclusive evidence for efficacy at reducing disease incidence or prolonging productive life of infected trees. For fungicides and fumigants to become a realistic method of disease control, more research is required to develop delivery systems for accurate application of the agents.

1.10.4 Resistance to *Ganoderma*

Progress on genetic resistance has been limited and research is complicated due to problems associated with the long breeding cycle of oil palm. Subsequent plantings are often subjected to different land preparation and management practices in order to minimise disease risk. Also, crop history has an effect on disease incidence in the field, making it difficult to determine resistance from other factors. Nevertheless one long-term study involved field observations of material of known origin in North Sumatra, Indonesia. *Elaeis guineensis* material of deli origin from Malaysia and Indonesia was more susceptible than African material (59), indicating possible genetic resistance within populations. However, the authors note the above considerations and point out the necessity for further research and development of an

effective screening process. Development of a rapid screen for resistance has been hampered by the requirement of a very large inoculum and several months for symptoms to manifest (154). To establish a successful screening programme, a smaller inoculum source and rapid infection is necessary before investigation of genetic resistance can be comprehensively investigated. One approach for the development of a rapid screening method has involved inoculation of oil palm at the germinated seed stage, which allows observation of symptoms after 3 months (31) and reports encouraging results in terms of differential susceptibility between crosses.

1.10.5 Biological Control

Little work has been done to study the potential of biological control of BSR in oil palm. Potential antagonists to *G. boninense* such as *Trichoderma* and *Penicillium* species have been isolated from Indonesia and Sumatra (1, 197, 217). Studies have shown antagonistic activity towards *Ganoderma* in culture but there are no reports of successful utilisation of biocontrol in the field. Biological control with regard to plant pathogens is discussed in chapter 3 and key points pertaining to biocontrol strategies for *Ganoderma* stem rot of oil palm are also described.

1.11 Related *Ganoderma* Diseases

The genus *Ganoderma* has a worldwide distribution and exists on all types of wood: gymnosperms, hard and softwood dicots and palms. Predominantly *Ganoderma* species exist as saprophytes but have been implicated in a number of diseases, primarily of palms. They are also implicated as diseases and parasites of trees in natural forests in South India (196).

1.11.1 *Ganoderma* on Coconut

Basal stem rot of coconut, also known as Thanjavar wilt, *Ganoderma* wilt, and Anabe Roga disease is a major problem in India and losses can reach as high as 31% (185). The causal agent has been named as *G. lucidum* and has also been reported as a parasite of palms in the Punjab and Sindh regions of Pakistan (153). A reddish brown viscous fluid oozes from the stem of infected palms, termed as bleeding. Drooping of

leaves, reduction in leaf production, reduction in leaf size and tapering are characteristic symptoms of the disease (26). Pathogenicity of isolated *Ganoderma* isolates was established by experimental infection of palms resulting in symptoms of disease (25). *G. applanatum* has also been isolated from infected palms but were unable to cause infection experimentally. *G. boninense* was reported to be responsible for the disease in Sri Lanka (170), but this was based on morphological characters that have been shown to phenotypically polymorphic. Therefore, taxonomic study on these isolates is required using molecular and cultural characteristics with interfertility studies for confirmation of species. Coconut in Malaysia and Indonesia are not considered susceptible to infection by *Ganoderma* spp. However, where poisoned or felled and replanted with oil palm *G. boninense* will colonise and sporulate from remaining logs and stumps, thus becoming a dangerous reservoir for *Ganoderma* isolates infectious to oil palm.

1.11.2 *Ganoderma* Diseases of Ornamental Palms

Ganoderma zonatum causes butt rot of numerous palm species in Florida in both landscaped and natural settings. The disease is never observed in tissue more than 4-5 feet from the base and is not observed in juvenile palms. The disease is identified by the presence of basidiophores at the base of infected palms and currently there are no means to identify infected palms other than appearance of basidiophores. It is believed that most palms are susceptible to the pathogen and basidiospores are the primary means of spread of the fungus, but has yet to be proven (68). Ornamental palms including oil palm are infected by *G. boninense* in Singapore (129). Disease pattern generally appears to be random and basidiospores have been implicated in dispersal and infection of the fungus.

1.11.3 *Ganoderma* Diseases of Rubber (*Hevea brasiliensis*), Tea (*Camellia sinensis*) and *Acacia magnium*

Ganoderma pseudoferrum (synonym *G. philippi*) causes red root disease of rubber and contributes to widespread root decay of rubber. Transmission of the fungus is believed to occur through root contact with inoculum in wood debris, although a number of insect species, in particular the tipulid flies, *Limonia nervosa* and *Limonia*

Umbrata, are regarded as potential vectors for the pathogen (141).

Lowland tea in Malaysia is also susceptible to attack from *Ganoderma* sp. and comparative studies on the morphology and physiology have determined that the species is closely related to *G. pseudoferrum* (240). Tea bushes are planted in close proximity and infection patches are spread through the stand by root-root contact. Infected bushes only show symptoms once infection has circled the collar region, with rapid death occurring during a dry period. On living bushes, the fungus only occurs as white mycelium with cherry red rhizomorphs and only produces basidiophores once the bush is dead.

Acacia magnium is indigenous to the far islands of Indonesia, the western province of Papua New Guinea and northeast Queensland, Australia. It grows rapidly and is cultivated in plantations throughout South East Asia for wood production. Two distinct root diseases are problematic to *Acacia* production: brown-root disease caused by *Phellinus noxius* and red-root disease thought to be caused by a *Ganoderma* sp. Pathogen identity in red-root disease is unconfirmed as no basidiophores are produced on infected living trees and attempts to stimulate basidiophore production in vitro have been unsuccessful (123). However, infected roots are covered by a wrinkled, reddish-brown mycelial mat that becomes very evident when the root is washed clean of soil, which is also characteristic of red-root disease in rubber. There are so far no reports of successful methods of control for these diseases and more taxonomic study is required for a definitive identification of the pathogens.

2 Infection and Epidemiology of *Ganoderma* Stem Rot

2.1 Introduction

Basal stem rot (BSR) remains the most common manifestation of *Ganoderma* stem rot on oil palm, with upper stem rot (USR) occurring much less frequently. There has been a general consensus within the industry that the primary route of infection is through root contact. Several other root-infecting fungal diseases of trees and perennial crops are spread by vegetative growth. Basidiospores from *Heterobasidion annosum* colonise cut pine stumps and progress into the dead roots. When living roots from surrounding trees contact colonised root tissue, infection of living tissue occurs and commonly results in a single genet (genetically identical mycelia) infecting trees over a wide area (255). *Armillaria* species cause serious root diseases in a wide range of woody plants and in Kenya they cause considerable damage in tea plantations. Infection by the fungus occurs primarily by means of rhizomorphs or by direct mycelial growth from diseased to healthy roots (159).

However, recent research using molecular analysis of *Ganoderma* isolates and vegetative compatibility groups, which indicate high genetic variability within cropping systems and led to doubts regarding the issue of epidemiology. Currently there are two principal views on the route of infection: i) Root outgrowth from oil palms encounter a source of inoculum, such as infected roots from adjacent palms or windrow material; these become infected and mycelial growth through the root towards the bole leads ultimately to rot of the base of the palm and basal stem rot disease; ii) Basidiospores are dispersed throughout the plantations by wind currents onto wounded palm surfaces leading to direct infection. Supporting evidence exists for both views, which serves to complicate the problem of disease control. For successful management of the disease it is pivotal that aetiology of the disease can be clarified.

2.1.1 Root Infection of Oil Palms

The primary evidence implicating vegetative spread of the disease is the ability to establish infection of containerised palm seedling roots by controlled inoculation and to re-isolate the fungus from disease lesions. Inoculation of oil palm roots with *Ganoderma* has been established since the 1960s. Navaratnam and Chee used very large 750 cm^3 blocks of naturally diseased oil palms tissue to inoculate oil palm seedlings. They obtained positive infection from 15 of 22 seedlings inoculated and reported that a large inoculum source was required to induce infections (154). However because of the non-axenic nature of the inoculum, the validity of the findings was questioned. Later Lim used a smaller inoculum of dikaryotic *G. boninense* mycelium grown on oat grains and obtained 100% infection of roots after 10 weeks (130). Lim stated that it was purity and quality of *Ganoderma* inoculum and not inoculum size that determine infection potential of inoculum. More recently an inoculation technique using colonised 750 cm^3 rubber-wood blocks was developed in Malaysia and provided successful infection of palm seedling roots (100). Sariah *et al* (199) also utilised a similar method to infect palm seedlings and obtained 100% infection of seedlings in Malaysia using colonised 5x5x15 cm rubber-wood blocks as an inoculum source. This method is now routinely used to infect seedling roots at Bah Lias research station, North Sumatra and was used in Singapore to test the pathogenicity of *Ganoderma* isolates obtained from basidiospores of diseased oil palms in Jahore and from ornamental palms in Singapore (129).

Infection of roots is also supported by field studies conducted in Sumatra by Hasan & Turner. Stumps left in the ground were more infectious than felled trunks left on the surface and buried shredded palm tissue was more infectious than shredded palm tissue left on the surface (99). Untreated stumps left in the ground resulted in 38% infection of seedlings adjacent to stumps from BSR palms compared with 6% infection in seedlings planted adjacent to healthy palm stumps. Furthermore, buried shredded oil palm material from infected oil palms showed greater infection than non-buried shredded material (40% versus 0%) after 2 years. Higher infection rates of seedlings adjacent to diseased stump and trunk tissue, suggests root contact as a likely route for infection. Also, it is probable that palm roots come into contact with buried colonised oil palm material more readily than with surface material. Similarly, a

long-term field trial in North Sumatra has shown high levels of seedling infection when planted adjacent to buried trunks. Seedlings showed 60% infection after 52 months when planted 0.5 m from buried trunks, 25% infection at 1 m distance from buried trunks and 0% infection at 1.5 m distance. All seedlings planted adjacent to non-buried trunks remained uninfected after this period (78).

Strong argument against the role of basidiospores in direct infection of oil palm comes from the absence of any reports of successful infection of palms using basidiospores as inoculum. Lim & Fong propose basidiospores as the infectious agent for natural infection of palms in Singapore with *Ganoderma*. However, they did not demonstrate infection using spores experimentally (129). So far there has been no reports of successful infection by basidiospores inoculum and attempts were also unsuccessful at Bah Lias research station, North Sumatra (77).

2.1.2 Direct Infection and Dissemination by Basidiospores

It has been proposed that basidiospores directly cause disease by germinating and colonising wounded tissue, specifically fronds, which leads to eventual penetration of the stem and disease formation (194). Despite the majority of stem rot infections occurring at the base of the stem there remains the unresolved issue of upper stem rot (USR). If root contact with an inoculum source is the route of infection, why do some palms show basidiophores in the upper reaches of the stem and as a result snap in the middle when decay undermines structural integrity? Stem rot is generally termed upper stem rot when basidiophores are located more than 1.5 m from the palm base and USR decay lesions do not extend from the palm root system. Upper stem rot lesions are sometimes associated with other fungi, notably *Phellinus* spp. (77) and this has been suggested as the initial trigger for USR infection, with *G. boninense* occurring as a secondary invader. This is unlikely however, since *G. boninense* is always associated with the disease and a variety of other fungi are only ever recovered sporadically.

Wound sites may provide an opportunity for infection by *G. boninense* basidiospores and mechanical damage by invertebrate pests such as *Oryctes rhinoceros* and rats is compounded by the nature of the crop itself. To harvest oil palm fruit it is necessary

to sever the peduncle (fruit stalk) and petiole (frond) to free the fruit bunch. Several harvests are made from one tree in a year, causing multiple large wound sites. A tough abscission layer forms at the base of a cut frond and likely poses a considerable challenge for infection progress. However, as the tree grows in height fruit becomes more difficult to harvest and the probability of wounding the trunk becomes greater. This may facilitate infection by allowing direct penetration of the pathogen into the palm trunk.

Evidence based on the discontinuity of USR from BSR infections and especially genetic diversity of *G. boninense* isolates within plantations suggests that basidiospores play a key role in the development of USR. Sanderson estimated spore production at 2 million spores released from a 100 x 50 mm bracket every minute (193). Since a single infected standing oil palm can have many *Ganoderma* basidiophores and even more are found on toppled palms there is likely to be very high numbers of basidiospores within plantations and these will inevitably contact wounded surfaces. Infection of wounded frond surfaces in particular was first established by Lim and Chee, who obtained infection of frond rachis with the same success rates as on roots using colonised oats as an inoculum source (130).

The role of basidiospores in epidemiology has been investigated by genetic analysis in Malaysia. Multiple *G. boninense* isolates from basidiophores from two locations were tested for somatic incompatibility (heterokaryon incompatibility) tests and mitochondrial DNA RFLP (mtDNA RFLP) was also performed to assess relatedness of isolates (143). Somatic incompatibility suggested more than one isolate of *G. boninense* existed within a single infected palm in 6 out of 8 palms tested. Furthermore, of the 39 isolates collected from one plot, 34 distinct somatic incompatibility groups were observed. Presence of multiple somatic incompatibility groups (SIG) within a relatively small area, on adjacent trees and even within single infected trees suggests spread of *Ganoderma* does not occur though radial growth of mycelium, but indicates spatially separated populations originate from a diverse initial inoculum. mtDNA RFLP analysis supported this conclusion, identifying a similar number of mtDNA RFLP groups from the same isolates, although the groups were not always in accordance with SIG groups. Genetic diversity within plantations has also been studied extensively within the Milne Bay Estates in Papua New Guinea and a

similar high level of genetic variability has been observed. Studies from this region have favoured basidiospores as the main cause of disease, describing disease lesions connected to cut frond bases through the vascular system. These authors consider root infection to be of minor importance (194). Data from mating studies and somatic (heterokaryon) incompatibility testing was in accordance with the findings of Miller *et al.* where high variation was found throughout the plantation. *Ganoderma* has a tetrapolar mating system (4) and strongly favours outcrossing. If monokaryons from the same bracket were to be crossed with one another only 25% would allow anastomosis and the formation of a dikaryon. In sexual compatibility studies it was found that there was great variation within the area studied, with 81A and 83B mating alleles identified, and the study found more genetic relatedness between isolates 15-17 km distant than between adjacent individuals (173). Somatic incompatibility studies yielded the same conclusions and showed that dikaryons from close proximity displayed strongly antagonistic reactions in culture with formation of pronounced demarcation lines, suggesting individuals with distinct genetic content (174).

Further observations on decorative palms in Singapore, including oil palm, with *Ganoderma* stem infections showed no obvious clustering of infections and concluded basidiospores to be the means for dissemination and infection (129). It has been suggested that *Ganoderma* may colonise tissues of the living oil palms saprophytically for some time and perhaps subsequent conditions of stress within the palm allows invasion of the stem tissues (48). Use of a *G. boninense* primer called GanET designed to specifically amplify the ITS2 region rDNA of *G. boninense*, achieved amplification within tissue of cut fronds on apparently healthy trees (32). Cut fronds were trimmed 0.25-1 cm to obtain a surface free from contamination and tissue was extracted for PCR amplification using a universal fungal primer ITS3 (251) and the *G. boninense* specific GanET primer. In 4/37 fronds tested amplification of the 321bp fragment was achieved (172) supporting the suggestion that spore colonisation of frond bases may be important in epidemiology. However, on subsequent analysis (after one year) of fronds that gave a positive result, a second positive was not always obtained and disease symptoms were not observed. Fronds testing positive using the GanET PCR probe are not necessarily colonised by living fungus, as PCR reactions have the potential to amplify DNA from both living and non-living cells. A possible flaw in the procedure is the depth at which testing was

carried out. Basidiospores are likely to be taken up by xylem vessels, which may extend further than 1 cm before reaching end walls. Therefore these results may be detecting inactive basidiospores at shallow depths within frond tissues as opposed to established colonies causing decay in cut fronds.

Basidiospores landing on and infecting cut frond bases or peduncles may not be the only candidates for USR infection and there has been little effort to determine whether insect vectors such as *O. rhinoceros* play a role in infection. Also Flood *et al* speculated that organic debris, which collects behind the frond axils could potentially allow growth of *Ganoderma* and provide an inoculum source for infection (77). These areas currently represent complete unknowns with regard to *G. boninense* infections of oil palm and will need to be addressed in order to determine the mode of infection by *Ganoderma*.

2.1.3 Molecular Characterisation of Fungi

Epidemiology of infectious diseases in humans has driven the development of sophisticated molecular tools, allowing subtyping of pathogenic organisms into strains and subtypes. Molecular characterisation of bacterial species is more advanced than in fungi for this reason, but many of the same techniques used in bacteria are also applicable for fungi. Choice of technique can be based on a number of factors including required sensitivity, facilities and skill levels within labs and cost (158). PCR-RFLP has been used to characterise fungal populations at the species level including *Fusarium* species from many geographic locations (62) and mycorrhizas in *Eucalypus* forests (85). This technique has mostly been superseded now as a result of reduced cost of direct sequencing of DNA. However, these techniques are mostly useful for interspecific comparisons as described for *Ganoderma* species above (see 1.3), but lack sensitivity to determine between strains. Randomly amplified polymorphic DNA (RAPD), which is based on random priming of DNA with short primers, has also been widely used to detect molecular variation in fungi including the entomopathogen *Metarhizium* (41) and for characterisation of monokaryons of *G. boninense* (176). The benefit of this system is that it does not require *a priori* knowledge of the target genome to be used. However, this technique is sensitive to

small changes within the laboratory environment, as use of arbitrary primers can result in inconsistent band amplification as a result of weak annealing by the primers, which can lead to problems interpreting gels. Amplified fragment length polymorphism (AFLP) is a similar fingerprint based technique developed by Vos *et al* (245). This technique first involves restriction digestion of the DNA and ligation of oligonucleotide adapters. Amplification of sets of restriction fragments by primers that extend into the restriction fragment provides selection of only certain fragments and these are resolved on denaturing polyacrylamide gels. AFLP has been used to investigate genetic variation in individuals and populations and has even been used to investigate genetic variation in *Ganoderma lucidum* after a space flight, although results here were inconclusive (178). This method similarly does not require prior knowledge of the target genome and produces more reproducible amplification than RAPD. However, this method is more labour intensive, is expensive and usually results in amplification of 50-100 restriction fragments. This technique can therefore be very powerful for fingerprinting of DNA of any origin or complexity (245), however, production of so many restriction fragments can be difficult and time consuming to resolve using gel electrophoresis and can make scoring of bands laborious in absence of an automated system.

A sequencing based typing method called multi locus sequence typing (MLST) was developed for use against bacterial human pathogens (213). This involves the sequencing of approximately ten housekeeping genes to characterise genetic diversity. MLST has become popular because it directly samples polymorphism present in nucleotide sequences and because each new study can use and add to all previously obtained data. The method has also been adapted to fungi, including *Candida albicans* and *Coccoides immitis* (223). This is a powerful phylogenetic tool, however it is not suited to phylogenetic studies in *Ganoderma* as currently available sequence data is not sufficient. Due to the difficulties associated with other fingerprinting methods and the inability to adapt MLST for sequencing of *Ganoderma*, randomly amplified microsatellites (RAMS) were used to characterise *Ganoderma* populations in oil palm during this study.

2.1.4 Randomly Amplified Microsatellites (RAMS)

Microsatellite is the commonest term used to describe tandem repeats of short sequence motifs, such as penta-, tetra-, tri-, and dinucleotide repeats, but are also known as simple sequence repeats (SSR) (260). Microsatellites range from 20-100 bp in length and are useful for evolutionary and genetic studies because of their inherent instability. Mutation rates in microsatellite loci in yeast is estimated to be 10^{-4}-10^{-5} compared with 10^{-9} for point mutations (223). High mutation rates during replication are mostly caused as a result of slip-strand mispairing (SSM) (65). The SSM process first involves the DNA polymerase 'slipping' during replication causing the template and newly replicated strands to become temporarily unaligned. The strands must then realign to continue replication. The repetitive nature of microsatellites often results in imperfect realignment since repeats can easily loop out of the DNA double helix. Looping of the template strand can result in deletion mutations resulting in shortened microsatellite sequences and looping of the replicating strand will result in additions, resulting in mutation rates at most microsatellites that are usually an order of magnitude higher than mutation rates at other loci within the same genome (65).

RAMS was first developed by Zietwicz *et al* who showed that species specific fingerprints could be obtained using oligonucleotide primers to amplify SSR sequences from a range of organisms including humans, dogs and fish (260). The method was first introduced to fungi by Hantula *et al* who proposed the RAMS acronym (91) and it combines the benefits of microsatellites with the universality of RAPDs. RAMS should produce fewer bands than RAPDs, as simple sequence repeats are present with relatively low frequency in basidiomycetes, and this should make resolution of gels easier. RAMS have now been used in a number of studies on fungi including diversity in isolates of *Phlebiopsis gigantea* from European countries (237), determining genetic variation between *P. gigantea* isolates from North America and Europe (236), analysis of genetic variation of pine rusts (*Cronartium flaccidum* and *Peridermium pini*) (92) and studies on virulence and diversity of the phytopathogen *Colletotrichum lindemuthium* (134). In addition, RAMS amplified fragments can be cut from the gel and cloned or re-amplified and sequenced in order to characterise microsatellite loci within fungi so as to develop multi locus microsatellite typing (MLMT), an adaptation of the MLST technique (223).

Conjecture and uncertainty surrounding mode of infection of oil palm by *G. boninense* is stifling attempts to develop adequate control strategies. To enable development of successful management practices, elucidating the route of infection is crucial. In this chapter, basidiospores, vegetative growth and other potential inoculum sources are investigated for their importance for spread and infection of the pathogen, *G. boninense*.

2.2 Materials and Methods

2.2.1 Sampling of Isolates

Isolates used in the study are shown in appendix, Tables 1a-c. *Ganoderma boninense* isolates GB1, BLRS1, GMB3 and GMR3 were used in infection studies. Isolate GB1 was obtained from a basidiophore at the base of a diseased oil palm and BLRS1 was obtained from rotting tissue at the edge of an advancing lesion of a diseased oil palm; both isolates were from Bah Lias Estate, North Sumatra, Indonesia. GMB3 was obtained from a basidiophore from an infected BSR palm and GMR3 was taken from the advancing edge of diseased tissue; both isolates were obtained from Gunung Malayu estate, North Sumatra. Isolation of *Ganoderma* isolates from rotting tissue was facilitated by use of *Ganoderma* selective medium (GSM). *Ganoderma* isolates used for molecular characterisation were also isolated from basidiophores and rotting tissue from these two estates and are displayed in Table 1.

2.2.2 Growth Media

2.2.2.1 *Ganoderma* Selective Medium (GSM) (15)

Antimicrobial and other components of the medium were made up separately as parts A and B respectively. The anti-microbial component (part A) consisted of 300 mg streptomycin sulfate, 100 mg chloramphenicol, 285 mg pentachloronitrobenzene (PCNB), 130 mg 25% Ridomil, 150 mg benlate T-20, 1.25 g tannic acid, 2 ml lactic acid and 20 ml 95% ethanol in 80 ml pre-sterilised distilled water. The non-antimicrobial component (part B) of the medium consisted of 5 g bacto-peptone, 0.25 g $MgSO_4$, $7H_2O$, K_2HPO_4 and 20 g agar in 900 ml distilled water and was autoclaved at 121°C for 20 min. Once part B had cooled to approximately 40-45°C, part A was added and mixed before pouring into petri dishes.

2.2.2.2 Potato Dextrose Agar (PDA)

After isolation, working cultures were maintained on potato dextrose agar (PDA) (Oxoid) in petri dishes. For long-term storage, cultures were stored on slants under oil or water at room temperature and also at 6°C.

2.2.3 Oil Palm Seedlings

Oil Palm seedlings used for infection studies were commercial Deli dura x AVROS pisifera crosses supplied by Bah Lias research station (BLRS), North Sumatra. Progeny from two crosses were used during the project: A1121/03-13 x BL148/05-08 and A1123/33-05 x BL148/05-08.

2.2.4 Preparation of Wood Blocks and Inoculation of Oil Palms

Rubber-wood and oil palm wood was obtained from Bah Lias estate, North Sumatra. Wood blocks were cut to varying sizes, dried at 70°C to kill most vegetatively growing organisms, rehydrated in warm (50°C) sterile distilled water (SDW) and then autoclaved for 1 h at 121°C. Blocks of defined sizes were inoculated using 1 cm^2 blocks of agar taken from the active edge of a *G. boninense* culture grown on PDA placed mycelium side down. Inoculated blocks were incubated at 28°C at 70% relative humidity (RH) for varying times, depending on the size of blocks to ensure complete colonisation of the blocks. Once fully colonised, blocks were applied to the roots of palm seedlings, covered in soil and placed in bags to avoid root contact with external inoculum sources. Seedlings were grown in the nursery at Bah Lias with monthly monitoring for symptoms of BSR.

2.2.5 Collection of Basidiospores and Preparation of Spore Suspensions

G. boninense basidiospores were collected in the morning by placing Whatman filter paper below the pores of an active fruiting body and held in place by aluminium foil. The paper was left in place for 3-4 h until the paper was thickly coated in dark brown basidiospores. Spores were then taken to the laboratory and left to air dry for 10-15 min if the filter paper was slightly damp. The spore-coated filter paper was then cut into small pieces and added to SDW (pH 5.5) to make a suspension. Spore concentration was calculated with a haemocytometer and in all cases spores were used on the same day as collection. To visualise xylem vessels for sectioning, 2 mg/ml eosin dye was added to the spore suspensions.

2.2.6 Scanning Electron Microscopy

Preparation of samples was conducted in a fume hood. Samples were collected and placed in 3.5% glutaraldehyde (agar scientific) in 0.05 M piperazine-*N*, *N* '-bis (2-ethanesulfonic acid) (PIPES) buffer at pH 8.0 to fix proteins. Tissue was then cut into 3x3x1 mm pieces and exposed to vacuum for 16-20 h to extract trapped air. Samples were then viewed using 'low temperature scanning electron microscopy' on a JEOL SEM6310 model scanning electron microscope fitted with an Oxford Instruments cryotrans 1500 system attachment.

2.2.7 Assessment of Xylem Vessel Length

A suspension containing distilled water, eosin dye and fluorescent vinyl particles was prepared and applied to the cut surface of oil palm fronds. The suspension was added to the cut surface of three different length fronds at 15 min intervals maintaining an excess of suspension on the cut surface at least for 1 h. The fronds were cut the following day and stored at 4°C. Thin sections were prepared every 1 cm using a razor blade and applied to slides for microscopy. Particles fluoresce red under UV and sections were examined on a Leica DMIRB microscope with fw4000 imaging software.

2.2.8 Biotester Air Sampling in Sumatra

A Biotest RCS centrifugal air sampler (Biotest UK) was used to sample the air concentration of spores within air in oil palm plantations. Water agar was loaded into the sampler and was run for 8 min. Agar blocks were then extracted and observed microscopically for trapped spores. Four samples were taken for each time point and location to provide mean concentration of spores/m^3 using the formula: cfu/m^3 air = # colonies on agar strip x 1000/volume of air sampled (in an 8 min period 320 litres of air was sampled).

2.2.9 DNA Manipulations

2.2.9.1 Harvesting of *G. boninense* Mycelia for DNA Extraction

Ganoderma was grown on PDA for 1 wk. 1 cm^2 plugs were taken from the leading edge of the mycelium and placed in 60 ml of 3% malt extract (Oxoid) in 250 ml conical flasks. These were incubated at 28°C on a rotary incubator at 120 rpm, for 4-5 days. Mycelium was then extracted from the medium, filtered and washed in SDW to remove residue. Mycelium was placed directly into liquid nitrogen for immediate freezing and ground to a fine powder in a mortar and pestle. 100 mg of the powder was then used for DNA extraction and the remainder was stored at -70°C for future extractions.

2.2.9.2 DNA Extraction

DNA extraction was achieved using either the CTAB-based method described below or with the Qiagen DNeasy® plant DNA extraction kit as described in manufacturer's instructions.

CTAB-based method (261).

100 mg of ground, frozen mycelium was transferred to a solvent-resistant centrifuge tube and 5 ml mercaptoethanol buffer was added. This was mixed by inverting and heated to 65°C for 30 min in a water bath to lyse cell walls. Five millilitres of chloroform:isoamyl alcohol (24:1v/v) was added and mixed gently by rocking until homogeneous. The mixture was then centrifuged at 11000 x g at room temp for 10 min and the upper aqueous layer was then collected with a wide bore pipette. DNA was precipitated by adding 0.54 (v/v) iso-propanol and collected by centrifugation at 11000 x g for 5 min at room temperature. Isopropanol was carefully drained from the pellet and resuspended in 2 ml TE buffer. When dissolved, 20 units of ribonuclease A was added followed by incubation at 37°C for 30 min. A 1:1 concentration of chloroform:isoamyl alcohol was then added and mixed gently before centrifugation at 11000 x g at room temperature for 10 min. The upper aqueous layer was then collected using a wide bore pipette. Ammonium acetate was added at 1:5 (v/v) to give a final concentration of 1.5 M ammonium acetate. Absolute ethanol was added to 2:1 (v/v) to ppt DNA and this was collected by centrifugation at 11000 x g for 5 min at room temperature. Ethanol was carefully discarded from the pellet and DNA re-dissolved in 500 µl of 200 mM ammonium acetate followed by a second

precipitation with 2 x vol absolute ethanol. The pellet was then dried under vacuum using a SAVANT svc200H speedvac and resuspended in 100-150 µl TE buffer.

2.2.9.3 Polymerase Chain Reaction (PCR) of Internal Transcribed Spacers ITS1& ITS2

Universal fungal primers ITS1 and ITS4 were designed from conserved regions of rDNA and were used to amplify ITS1, 5.8S gene of rDNA and the ITS2 of *G. boninense* (251). The primer sequences are shown below and yield a product of approximately 650 bp (122). ITS1 primer (5' TCCGTAGGTGAACCTGCGG) is from the 3' end of the nuclear small subunit rDNA (18S RNA) and ITS4 (5'TCCTCCGCTTATTGATATGC) is from the 5' end of the nuclear large subunit rDNA (28S RNA). PCR amplifications were performed on a MJ research (model PTC peltier thermal cycler). Reactions were carried out in a total volume of 50 µl consisting of 0.4 µM of each primer, 50 mM KCl, 10 mM Tris-HCl (pH9.0 at 25°C), 1.5 mM $MgCl_2$ and 0.1% Triton® X-100, 1U *Taq* DNA polymerase (Promega) and 20 ng of *G. boninense* genomic DNA. Amplification cycles were performed with an initial denaturation of 5 min at 95°C followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C and 3 min extension at 72°C with a final 10 min extension at 72°C. To determine success, PCR products were run on a 1.7% w/v agarose gel and stained with 1 µl/10ml ethidium bromide.

2.2.9.4 Cleaning PCR Products

PCR products were cleaned using the column based Qiagen QIAquick® PCR Cleanup Kit as described in manufacturer's instructions for individual samples. Polyethylene glycol (8000 mw PEG) was used for cleanup of large-scale (96 well PCR plates, AB Gene) PCR reactions. 60 µl of a 200 g/l 8,000 mwt PEG and 146 g/l NaCl solution was added to each well before sealing with PCR film (AB Gene) and centrifuging at 400 x g for 20 sec before incubating at room temperature for 30 min. The plate was then centrifuged for 30 min at 1350 x g and 4°C. The seal was then removed, blotted onto blue roll and centrifuged inverted on blue roll at 1350 x g for 20 sec. 150 µl of chilled 70% ethanol was then added to each sample and centrifuged at 1350 x g for 30 min at 4°C. The seal was again removed and blotted on blue roll and then centrifuged inverted on blue roll at 200 x g for 20 sec. The plate was then placed onto a PCR block programmed for a temperature of 37°C for 2 min to

evaporate ethanol. Twelve microlitres of milli Q water was added to each well, vortexed then centrifuged at 400 x g for 20sec. Vortexing and centrifugation was then repeated three times to fully dissolve nucleotides. Success of the cleaning procedure was observed by running a 2 µl sample from each well on a 1.7% w/v agarose gel and staining with 1 µl/10 ml ethidium bromide.

2.2.9.5 Sequencing Reactions

Sequencing reactions were performed in 5 µl volumes using 96 well PCR plates (AB Gene). Two microlitres of cleaned PCR products were added to each well, 1 µl of forward primer was added into 1 well of each sample and 1 µl of reverse primer was added into another well of the same sample. Two microlitres of Bigdye® (Applied Biosystems) was then added to each well before sealing the plate, centrifuge at 400 x g for 20 sec. Reactions were performed on a MJ research (model PTC peltier thermal cycler) thermal cycler with an initial denaturation of 5 min at 95°C followed by 25 cycles of 96°C for 30 sec, 5 sec annealing at 50°C, 4 min extension at 60°C as described in the manufacturer's instructions.

2.2.9.6 Cleaning Reactions

Twelve millilitres of miliQ water was added to each well, sealed, vortexed and centrifuged at 400 x g for 20 sec. Fifty-two microlitres of absolute ethanol and 3 mM sodium acetate (50:2) was then added to each well and mixed. The plate was then chilled in a -20°C freezer for 30 min before centrifugation at 1350 x g for 30 min. The plate was then blotted onto blue roll and centrifuged inverted on a blue roll for 20 sec at 200 x g. 150 µl of 70% ethanol was added to each well, sealed and centrifuged at 1350 x g for 30 min. After centrifugation the plate was blotted on blue roll and centrifuged inverted on blue roll at 200 x g for 20 sec. The plate was then left on the bench top for 30 min to air dry before sealing before sending for sequence analysis.

2.2.9.7 DNA Fingerprinting

Fingerprinting of *G. boninense* was carried out using randomly amplified microsatellites (RAMS) as described (50). Degenerate primers 5'DHB$(CGA)_5$ and 5'HBH$(GAG)_5$, where D=A/G/T, H=A/C/T, B=C/G/T, were used to amplify microsatellite DNA. PCR amplifications were performed on an MJ research (model PTC peltier thermal cycler) in a total volume of 50 µl consisting of 0.4 µM of each primer, 50 mM KCl, 10 mM Tris-HCl (pH9.0 at 25°C), 1.5 mM $MgCl_2$ and 0.1%

Triton® X-100, 1U *Taq* DNA polymerase (Promega) and 20 ng of *G. boninense* genomic DNA. Reactions were performed with an initial denaturation of 10 min at 95°C followed by 37 cycles of 30 sec denaturation at 95°C, 45 s annealing at 61°C and 2 min extension at 72°C, followed by a final 10 min extension at 72°C. Observation of bands was achieved by running 20 µl of the reaction mixture on a 2% w/v agarose gel at 50 v in a large gel tank to obtain clear band separation.

2.2.10 Statistical Analysis

At least three RAMS amplifications were performed for each *Ganoderma* isolate and only amplicons that reproduced consistently were scored for presence (1) or absence (0). Identical banding patterns were regarded as genetically identical and were only introduced to the matrix once for statistical analysis. The reduced matrix was analysed using cluster analysis based on standardised Euclidean distance (78) and using the unweighted pairgroup method with arithmetic means (UPGMA) (84) to investigate genetic relationships using MVSP package (Kovach Computing, Anglesey).

2.3 Results

2.3.1 Potential of Dikaryons and Monokaryons to Cause Infection

Ganoderma can exist as monokaryotic or dikaryotic mycelium; the potential of the two forms to infect roots was investigated. Dikaryotic *Ganoderma* isolates were obtained from infected palm tissue using *Ganoderma* selective medium (GSM). Isolates were observed for presence of clamp connections (by light microscopy indicative of dikaryotic mycelium). Basidiospores were obtained from a basidiophore attached to the base of an infected oil palm at Bah Lias research station, North Sumatra. Spores were prepared as a $1x10^5$ spores/ml suspension in SDW, pH5.5, and 1ml was pipetted onto PDA plates. After two days the plates were examined by light microscopy and well-segregated colonies were sub-cultured onto fresh PDA petri dishes. The plates were then left for five days and monokaryons identified by absence of clamp connections.

Dikaryotic and monokaryotic mycelia were then inoculated separately onto 10 12x6x6cm rubber-wood blocks and left for 9 weeks to fully colonise. Rubber-wood was used as an inoculum source as these induce greater infection of seedlings than colonised oil palm wood. Fully colonised blocks were placed in direct contact with the roots of ten 6 month old seedlings. Ten uninoculated blocks were placed in contact with oil palm seedling roots as negative controls. The seedlings were then grown in the nursery at BLRS with monthly monitoring for symptoms of BSR.

First infection symptoms were observed after 4 months (Fig. 7) when three seedlings inoculated with dikaryotic mycelium began displaying characteristic one-sided mottling of fronds. Levels of infection increased over the subsequent seven months until 80% of seedlings were infected. By 15 months, all symptomatic seedlings inoculated with dikaryotic mycelium had died, whilst all seedlings inoculated with monokaryotic mycelium remained asymptomatic. The plants were then split longitudinally to score internal symptoms. Those plants infected with dikaryotic mycelium had characteristic decay of the basal stem tissue while those inoculated with monokaryotic mycelium had no decay and appeared similar to controls. Thus,

monokaryotic mycelium (from basidiospores) failed to induce symptoms in young seedlings while wood colonised by dikaryotic mycelium was successful.

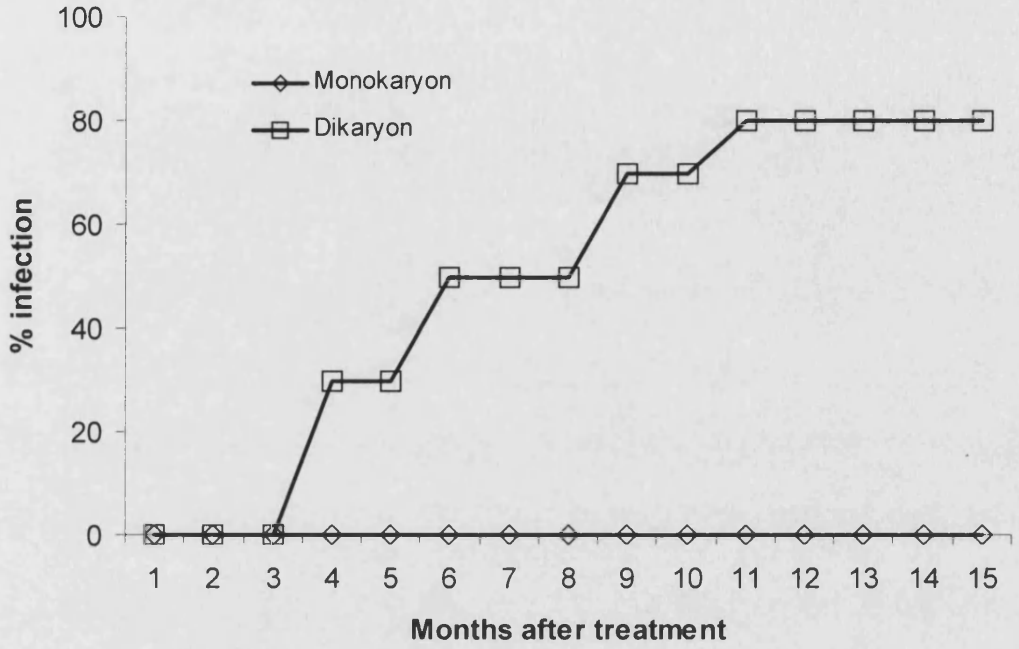

FIG. 7. **Infection of oil palm seedlings by dikaryotic and monokaryotic mycelium.**

2.3.2 Effect of Inoculum Size on Infection

Blocks of defined sizes were inoculated using 1 cm^2 blocks of agar taken from the active edge of a *G. boninense* culture grown on PDA. Inoculated blocks were incubated at 28°C at 70% relative humidity for varying times depending on the size of blocks to ensure complete colonisation of the blocks by the fungus: the 12x6x6 cm blocks were incubated for 9 weeks, 12x3x3 cm blocks incubated for 7 weeks, 6x3x3 cm blocks incubated for 5 weeks, 3x3x 3cm blocks incubated for 4 weeks, 3x1x1 cm was incubated for 3 weeks and 1x1x1 cm blocks were incubated for 1 week. Uninoculated 12x6x6 cm control blocks were used as negative controls. Once fully colonised, blocks were placed in direct contact with the palm roots of 6 month old seedlings and placed in bags to avoid root contact with external inoculum sources. Seedlings were grown in the nursery at Bah Lias and monitored monthly for symptoms of BSR.

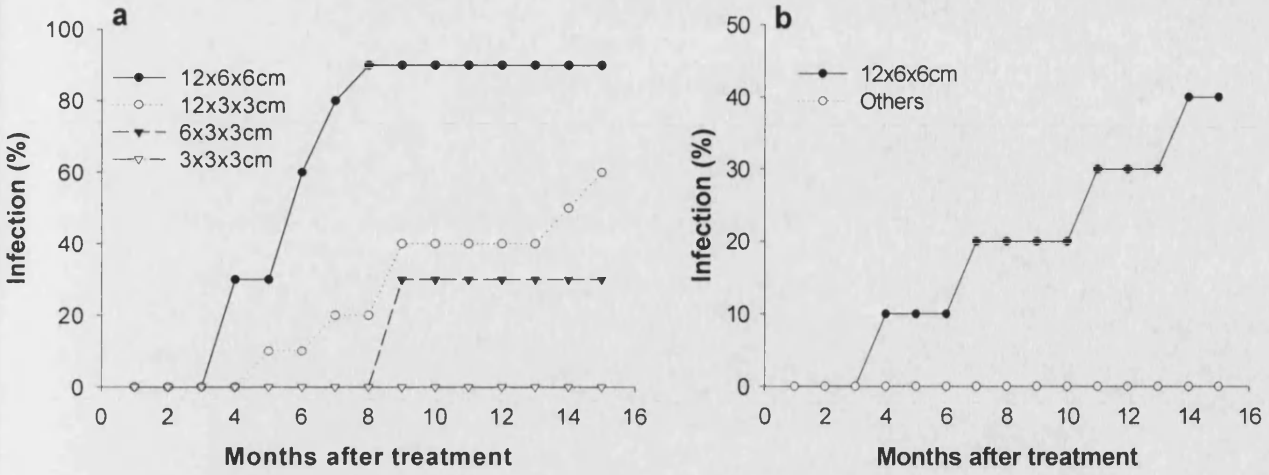

FIG. 8. **Effect of different sized inoculum sources on infection.** Each time point shows percentage infected seedlings out of a total of ten replicate seedlings. **a** Colonised rubber-wood blocks used as inoculum source. Analysis for relationships using chi-square (χ^2) analysis showed significant differences in frequency of infection between inoculum sizes ($P < 0.001$, df =2). Most infection occurred after inoculation with colonised 12x6x6 cm blocks ($P < 0.001$, df =1) followed by 12x3x3 cm blocks, which induced significantly greater infection than colonised 6x3x3 cm blocks ($P < 0.0187$, df = 1). **b** Colonised oil palm wood blocks used as inoculum source. Only 40 percent of seedlings were infected after 16 months by the largest colonised oil palm wood block. Colonised blocks smaller than 12x6x6 cm induced no infection.

Infection of oil palm seedlings was found to be greatest when a large inoculum source was used (Fig. 8). The largest inoculum source used in the study was 12x6x6 cm, which induced 90% infection after 8 months from colonised rubber-wood blocks. Infection was progressively lower depending on inoculum size, 60% infection by 12x3x3 cm blocks and 30% by 6x3x3 cm blocks respectively after 15 months. Most infection began 8-9 months after inoculation and few additional cases of infection occurred after this time. However, palms inoculated with the 12x3x3 cm blocks showed an additional 20% infection between the 13-15 m period. Late infection may be because roots were not in direct contact with the inoculum source in the initial stages but roots grew towards the inoculum source subsequently. No blocks smaller than 6x3x3 cm induced infection. Colonised oil palm blocks had reduced potential to induce infection with only 40% infection after 15 months in seedlings inoculated with 12x6x6 cm blocks and no infection in seedlings treated with smaller inoculum sources. These results indicate that the rate of infection is quicker with increasing size of inoculum and that colonised rubber-wood has greater inoculum potential than colonised oil palm wood, under controlled conditions.

2.3.3 Effect of Shading on Infection Potential of *Ganoderma*

The possible influence of shading on *G. boninense* infection of oil palm seedlings was studied by field trials in Sumatra. Twenty 6 month old oil palm seedlings were inoculated with 12x6x6 cm rubber-wood blocks colonised by *G. boninense* and twenty seedlings were left uninoculated. Ten of the inoculated palms and ten controls were placed in shaded conditions whilst the remainder were exposed to sunlight. Monthly assessment of symptoms was made over fifteen months. Measurements of soil temperature were made from the centre of the seedling bags with a thermometer over a three-week period and average temperatures calculated for daylight hours.

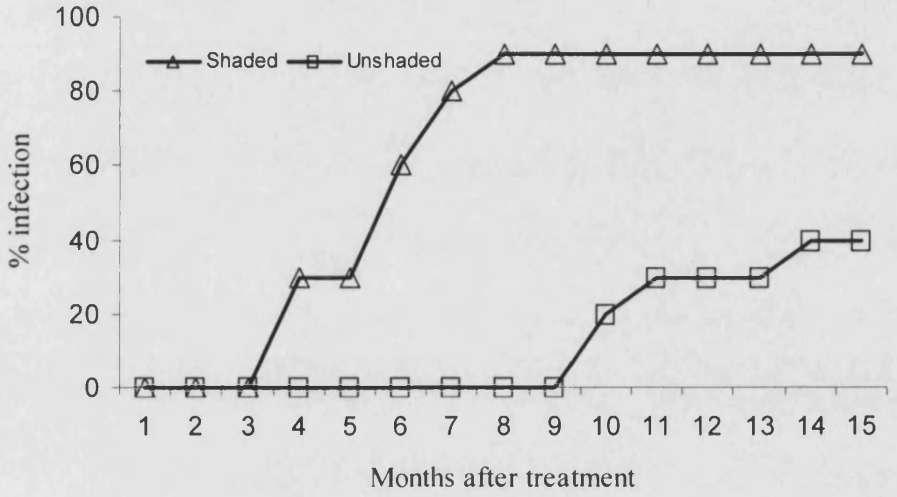

FIG. 9. Effect of shading on infection. Each observation represents the percentage of infected seedlings from 10 replicates at a particular time point. Infection of oil palm roots was significantly greater under shaded conditions compared to non-shaded conditions ($P < 0.0001$, df = 1) by chi-square (χ^2) analysis.

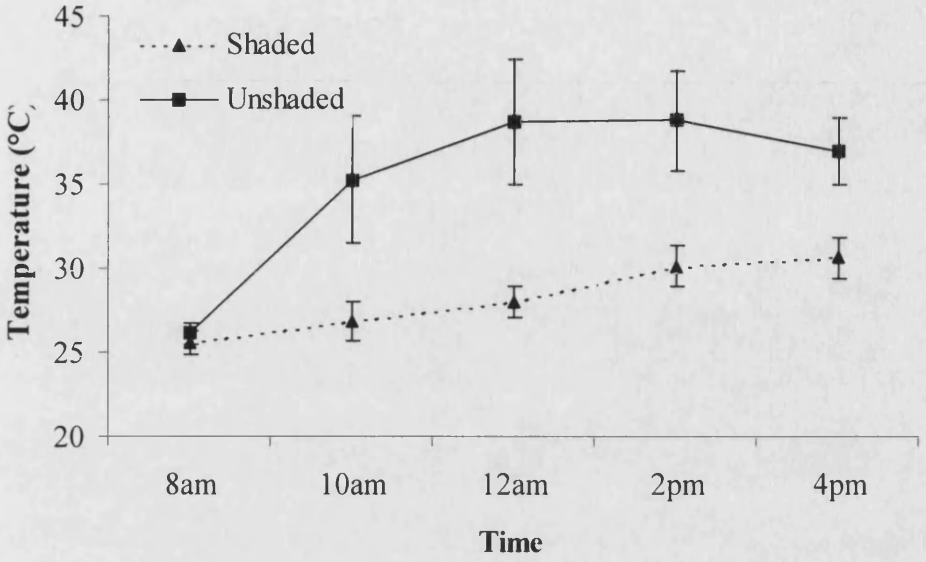

FIG. 10. Temperature of soil from seedlings during daylight hours under shaded and non-shaded conditions. Soil temperature under shaded conditions was significantly lower than non-shaded soil ($P = 0.0001$) by comparison with Mann-Whitney U test. Error bars represent standard deviation of mean from seven measurements for each time point.

Efficacy of *G. boninense* to infect oil palm seedlings was markedly reduced in direct sunlight by comparison to shaded conditions (Fig. 9). After 8 months 90% of shaded seedlings were symptomatic compared to 0% infection of un-shaded palms over the same period. Un-shaded seedlings began to show symptoms after 10 m and percentage of seedlings showing symptoms had risen to 40% by 15 months. Temperature of soil from un-shaded seedlings was highest at midday and averaged 37°C at midday (Fig. 10). Temperatures were much greater in direct sunlight and soil temperatures rose above 40°C on more than 50% of the observed days; the highest recorded temperature was 45°C. Temperatures were more consistent in soil from shaded conditions, and did not exceed 32°C (Appendix, Table 2a&b). This is because the average temperature also includes data from overcast days when soil temperatures were considerably lower.

2.3.4 Effect of Temperature on Hyphal Extension *in vitro*

In view of the marked differences in soil temperature between shaded and exposed conditions and the corresponding severity of infection, the effect of temperature on *Ganoderma boninense* growth and viability was assessed. Isolates BLRS1 and

GMR3 were grown on PDA agar for five days at 28°C then 1 cm^2 sub-cultured onto PDA. Three measurements were made from each plate daily and the mean was used to give an estimate of hyphal extension in millimetres/day for each temperature. Hyphal extension was measured at 25, 30, 36, 40 and 45°C.

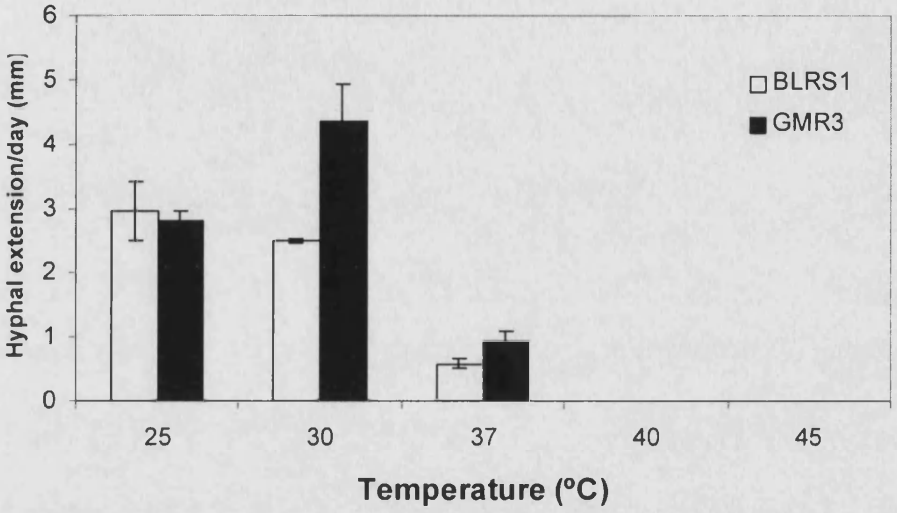

FIG. 11. **Effect of temperature on hyphal extension of two pathogenic strains of *G. boninense*.** Hyphal extension was recorded after 2, 7, 9 and 14 days and used to calculate mean growth rate/day.

Ganoderma boninense grew well at temperatures between 25 and 30°C (Fig. 11 & Fig. 12). Growth was reduced considerably at 35°C (Fig. 12) but mycelia remained healthy and if the plate was returned to 28°C, maximum growth rate resumed. At 40°C no *G. boninense* growth was observed. There was an initial burst of hyphal extension during the first day of incubation but no further growth occurred. However, when cultures were returned to 28°C, recovery of hyphal growth was possible. At 45°C, no growth was observed for *G. boninense* and no recovery was possible after exposure to this temperature for 2 days. Therefore, *G. boninense* appears to be unable to survive at temperatures of 45°C or more under laboratory conditions and such extreme temperatures, as encountered in exposed soil are likely to pose substantial stress or even be fatal to the pathogen under natural conditions.

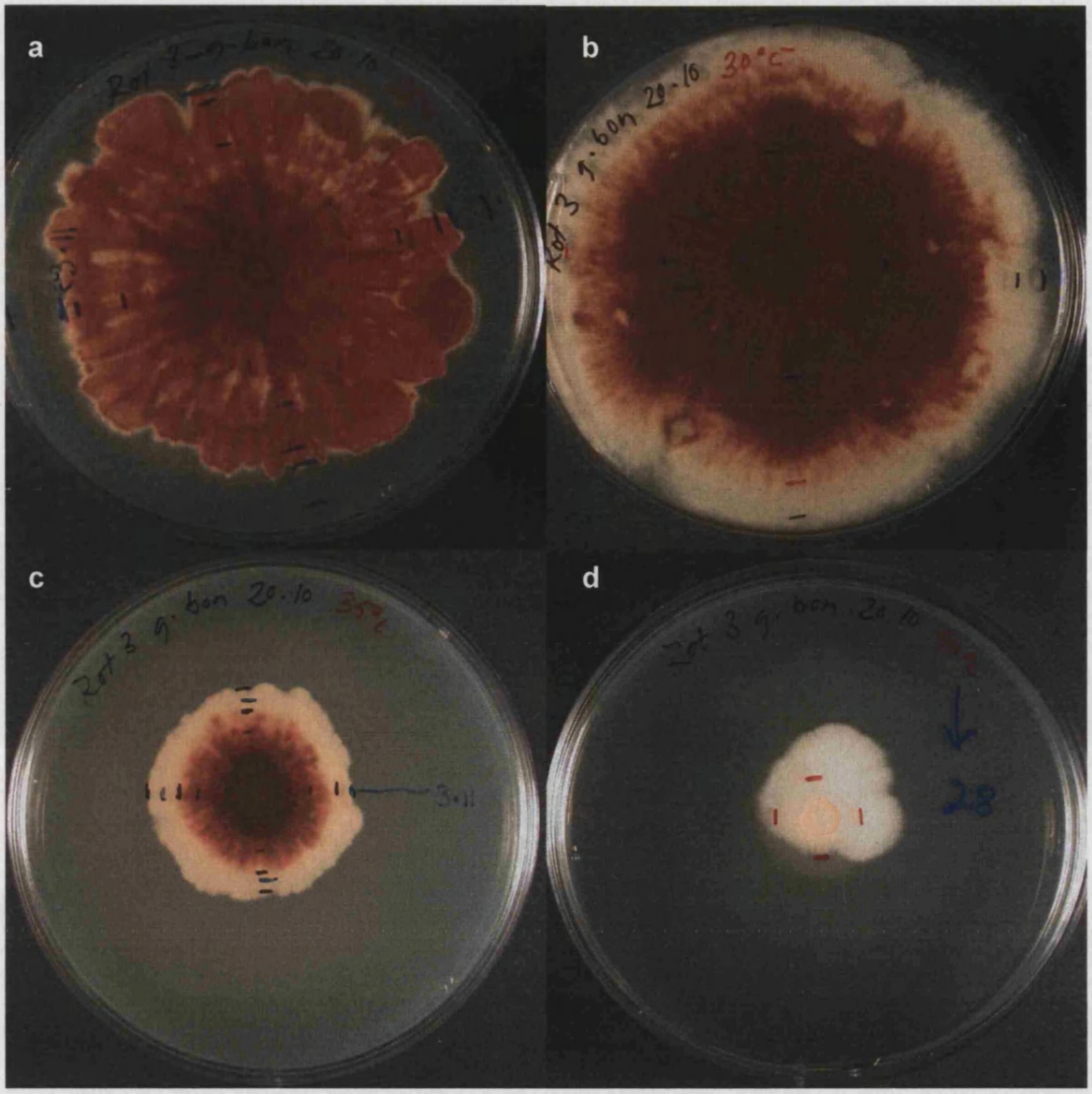

FIG. 12a-d. Hyphal extension of *G. boninense* at 25, 30, 35 and 40°C. **a** Hyphal extension at 25°C, note how mature mycelium becomes melanised and the agar becomes ruffled. **b** Growth at 30°C, melanisation and agar ruffling is reduced compared to mycelia at 25 °C. **c** Growth of *G. boninense* at 35°C, note reduced growth rate. **d** Hyphal extension at 40°C. Red lines indicate where growth was halted at 40°C subsequent growth is due to subsequent recovery at 28°C.

2.3.5 Root Infection Using Small Inoculum Source

As previous experiments in BLRS had indicated that small inoculum was not sufficient to induce symptoms in 6 month old seedlings after 15 months, it was decided to test this further under greenhouse conditions in the UK. If infection could be induced rapidly using a small inoculum source, this would be invaluable to study the host-parasite interaction as a potential practicable screen for disease resistance.

Three isolates of *Ganoderma boninense*: BLRS1, GMB3 and GMR3 were grown on PDA agar in the dark for 7 days and 1 cm^2 agar from advancing mycelia were used to inoculate wood blocks (3x3x3 cm). Both oil palm and rubber-wood blocks were incubated from a small inoculum source and infection of both wounded and non-wounded roots was clearly demonstrated (Fig. 13). Half of the inoculated oil palm and rubber wood blocks were used to inoculate roots wounded by slicing a 1 cm^2 section from the root epidermis, the remaining bocks were used to inoculate unwounded roots. Inoculated blocks were incubated in sterilised, Nalgene 125 ml polypropylene containers at 28°C, in dark conditions for one month to allow complete colonisation of the wood. Three millilitres of water was added to the containers to maintain high humidity levels. Blocks were raised out of the water by placing on sterilised plastic rods. These were then used to infect healthy 18-month-old oil palm by attachment to individual roots with parafilm. For each isolate there were four treatments: I) colonised rubber-wood blocks attached to wounded roots, II) colonised rubber-wood blocks attached to non-wounded roots, III) colonised oil palm blocks attached to wounded roots, and IV) colonised oil palm blocks attached to non-wounded roots. For each treatment four 18-month old palm seedlings were used and five spatially separated roots were inoculated with *Ganoderma* on each palm. Once blocks were attached to the roots the plant was re-potted into large (10 litre) pots and grown at 28°C and 70% relative humidity. Examination of inoculated roots was made monthly and an individual root was harvested after each month. Positive infection was determined by isolation of *Ganoderma* on GSM from internal root tissue after surface sterilisation using 1% Chloros. Estimation of infection progress was made by taking samples of root at 1 cm intervals and placing onto GSM to re-isolate the fungus.

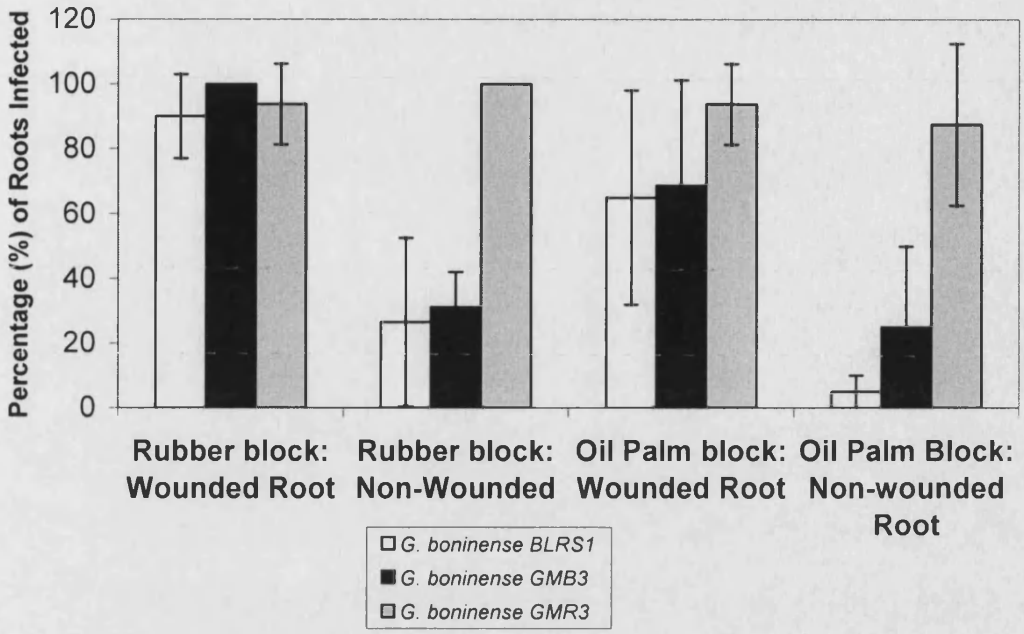

FIG. 13. Greenhouse infection of oil palm seedlings using rubber-wood and oil palm-wood blocks in the presence and absence of root wounding. Error bars represent standard deviation of means from roots of five inoculated seedlings. Data from all observations of root infection by GMR3 was pooled and compared statistically with pooled infection data from BLRS1 and GMB3. Significant differences in aggressiveness were observed in comparison with GMB3 ($P < 0.001$, df = 1) and BLRS1 ($P < 0.001$, df = 1) using chi-square (χ^2) analysis, isolate GMB3 was significantly more aggressive than BLRS1 ($P < 0.031$, df = 1). Pooled infection frequencies of wounded roots were significantly higher than non-wounded roots ($P < 0.001$, df = 1). Rubber-wood blocks were shown to induce greater infection of wounded roots than colonised oil palm blocks ($P < 0.006$, df = 1).

Infection was clearly demonstrated from a small inoculum source in this study (Fig. 13). The experiment also highlighted variability in pathogenicity between the *Ganoderma* isolates; GMR3 was the most aggressive pathogen and caused infection in over 80% of seedlings for all treatments. Over 80% infection was found from all isolates when colonised rubber blocks were used as inoculum to wounded roots. When rubber-wood blocks colonised with *G. boninense* were attached to a wounded root, infection rates were over 90% for all 3 isolates of *Ganoderma* (Fig. 13), however, isolates BLRS1 and GMB3 were found to be less aggressive when inoculated to non-wounded roots or when oil palm blocks were used as inoculum sources (Fig. 13). BLRS1 was shown to be the weakest pathogen by chi-square analysis. *G. boninense* GMR3 was also shown to have the most rapid progression through the root and infection progressed at an average of 4.4 cm/month (Fig. 14), which was significantly faster than GMB3 (2.1 cm/month) and BLRS1 (2.3

cm/month). Use of colonised rubber blocks against wounded roots was shown to have the greatest efficacy for infection, followed by colonised oil palm blocks on wounded roots and wounding was shown to aid infection by the weaker pathogens.

It is clear, therefore, that a small inoculum source could successfully induce infection of oil palm roots. Establishment of infection was successful in all cases using 3x3x3 cm oil palm blocks when the inoculum was physically attached to the roots. Thus it appears that absence of infection by blocks of the same size when not physically attached to roots (2.3.2) suggests that intimate association with the pathogen is more important than inoculum size. In addition, there are clearly differences in aggressiveness between isolates and this can be circumvented if roots are wounded. Finally, rubber-wood was shown to be a better inoculum source for infection of oil palm roots, consistent with previous experimental results and findings of other researchers.

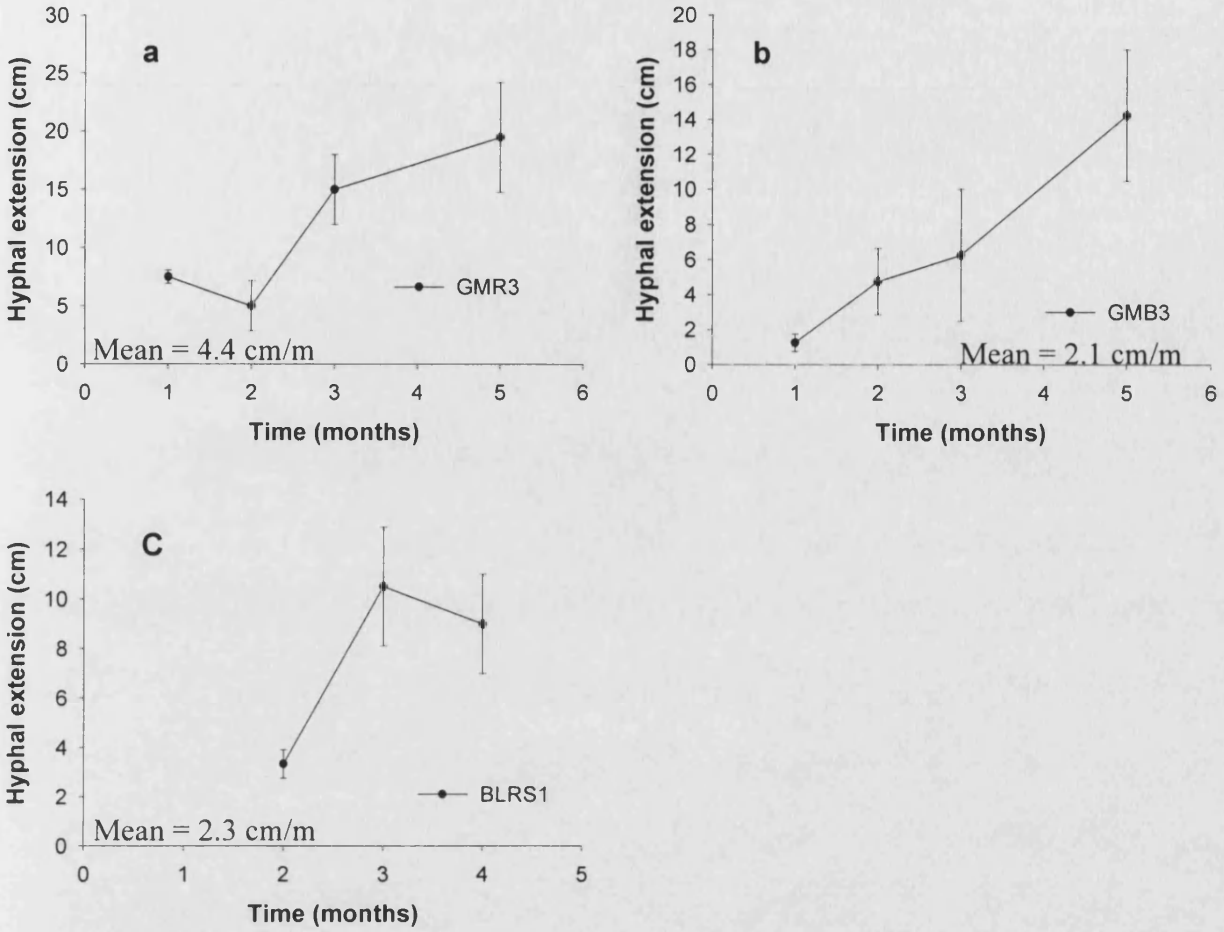

FIG. 14a-c. Advance of infection within infected roots of oil palm seedlings. a *G. boninense* isolate GMR3 progressed most quickly along the root. After 5 months most of the root infections progressed to the bole. **b** Progress of infection by *G. boninense* GMB3 was quite slow and average monthly extension within the root was less than half that of GMR3. **c** BLRS1 had a similar growth rate within the root as GMB3, which was much slower than GMR3. One-way ANOVA showed that mean hyphal extension/month was not the same between isolates ($P < 0.0004$, df = 2) and comparison of means by Tukey-Kramer HSD showed that hyphal extension of GMR3 was significantly faster than GMB3 and BLRS1 ($P = 0.05$), however, there was no significant difference between hyphal extension of GMB3 and BLRS1 ($P = 0.51$).

2.3.6 Macroscopic Examination of Infection

Root infection occurs after intimate association with the inoculum source. For infection of seedlings *in vitro*, contact was affected by physically strapping the inoculum source to suitable primary roots. Penetration of the root epidermis and exodermis occurred, presumably as a result of cell wall degrading enzymes (CWDE). Sometimes hyphae grew around the surface of the root, forming stroma-like tissue (thick cell walled, melanised mycelia) (Fig. 15a). Where infection was successful,

mycelial growth by the fungus resulted in the wood block becoming firmly attached to the root. From the point of infection, *G. boninense* grew steadily throughout the root, degrading host tissues. Heavily infected tissue within the root was evident by brown discoloration, primarily in the cortex. However, the advancing edge of infection was determined by use of GSM to isolate *G. boninense* from the infected tissue (Fig. 15b). Using this method it was possible to follow the rate of root colonisation by the fungus, although sometimes infection could be seen as *G. boninense* emerged from infected root tissue by breaking through the epidermis (Fig. 15c). As infection progressed into the bulb (bole in mature palms), infected tissue appeared brown and the perimeter of this region was often a dark brown and has been termed the 'reaction zone' (51). Immediately in front of the infected area is a small area of yellow tissue, termed the 'yellow zone'. Heavy colonisation of the basal tissue interrupts the vascular system, presumably leading to water stress and foliar symptoms. Complex vascular tissue permeates the lower stem of oil palm, which can compensate for substantial decay, however, eventually the entire stem becomes decayed, leading to palm death.

2.3.7 Infection of Mature Palms

Random felling of asymptomatic palms from different aged plantings showed that most palms remain healthy until ten years. The bole of all sampled palms below 12 yrs had unblemished, healthy, pale-yellow wood (Fig. 16a). After 12 yrs, evidence of BSR infection became more prevalent and Fig. 16b shows multiple brown rotting lesions on the bole tissue after the roots had been excised. Lesions clearly progress from infected roots (Fig. 17a&b) and establish in the bole tissue. Lesions coalesce to cover much of the bole tissue (Fig. 18), coinciding with foliar symptoms and basidiophore formation. Since multiple roots appear to be infected, it is possible that the palm may be simultaneously infected by more than one isolate of *G. boninense*.

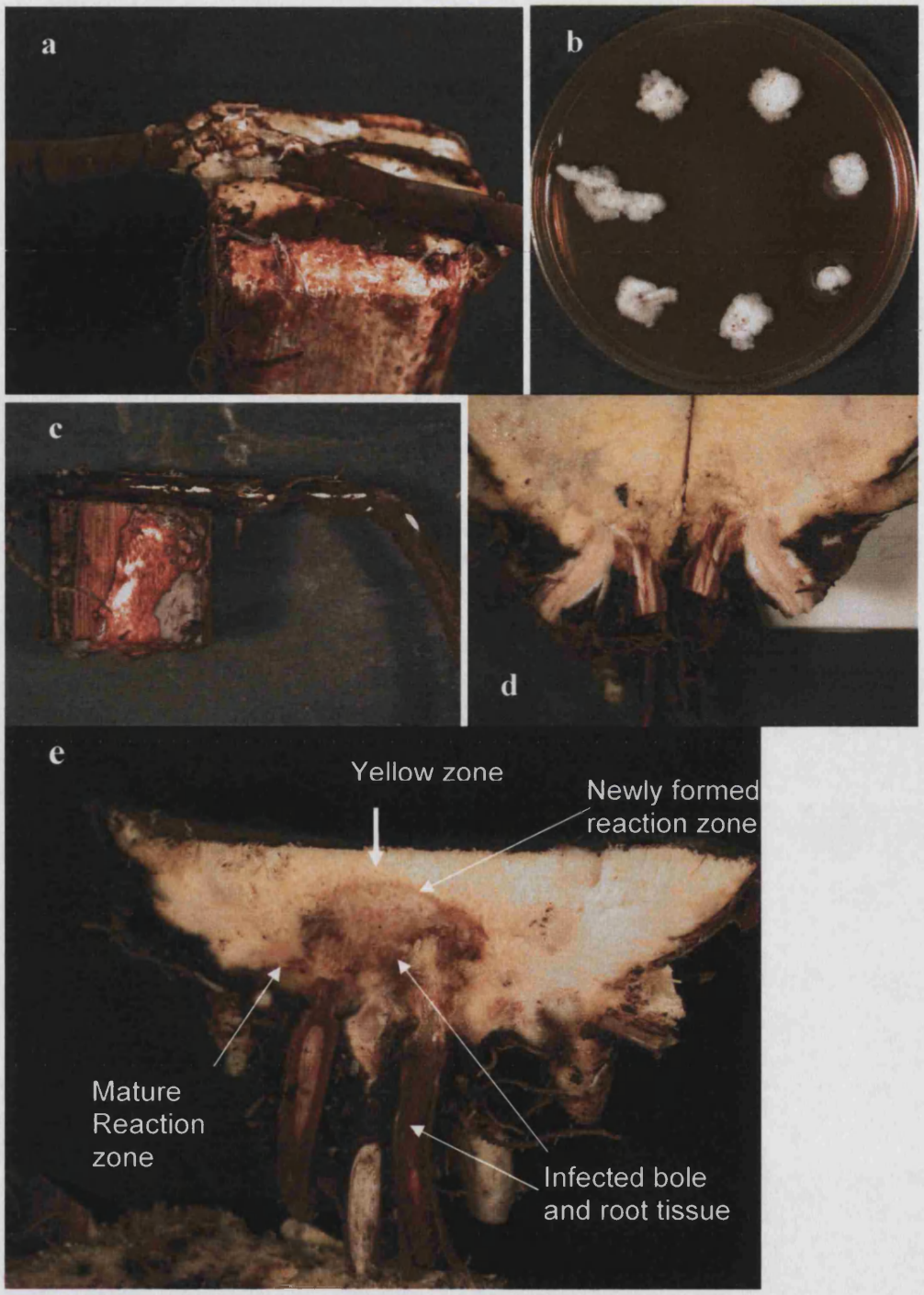

FIG. 15. Infection of oil palm seedling roots and manifestation of disease in the bole by *G. boninense*. **a** Rubber-wood block used as inoculum source firmly attached to an oil palm root with mycelial growth around the root (arrow). **b** Re-isolation of *G. boninense* on GSM. **c** *G. boninense* mycelium protruding through the epidermis from infected roots. **d** Progress of infection to the root/bole interface. **e** Advance of the fungus into the bole tissue. Tissue appears brown and the perimeter of this region is often a dark brown and termed the 'reaction zone'. Immediately in front of the infected area is a small area of yellow tissue, termed the 'yellow zone'. Uninfected tissue is white or pale yellow.

FIG. 16. **Bole of healthy and infected 12yr old oil palms. a** Pale-yellow uninfected bole tissue. **b** Brown mottling caused by decay lesions emanating from multiple root infections.

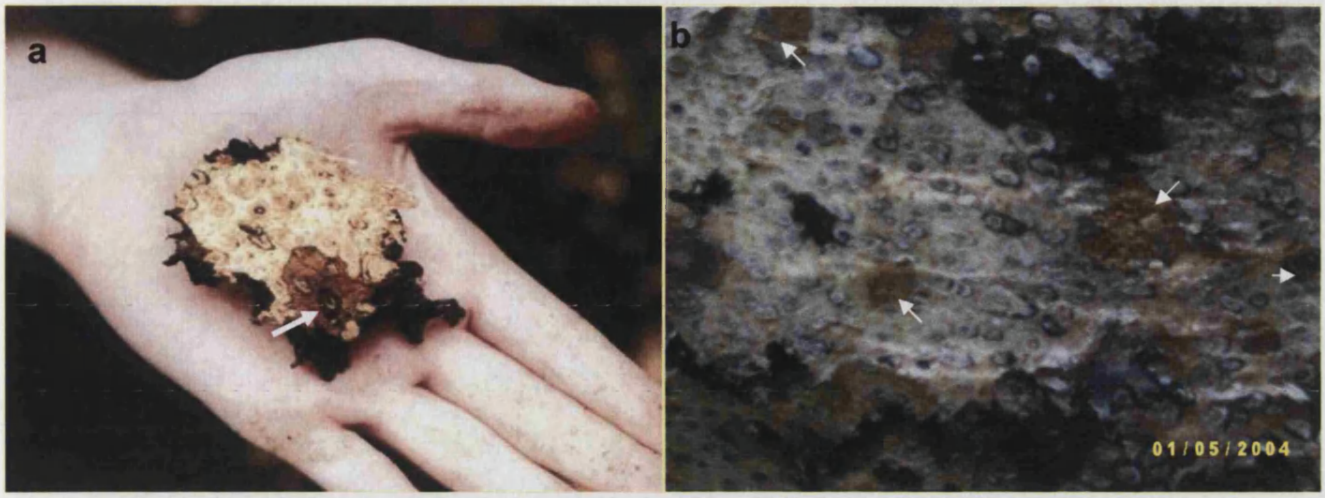

FIG. 17. Images of early decay lesions. **a** Infection seen clearly progressing from a heavily infected root with a clearly defined perimeter. **b** multiple decay lesions on a tree suffering from subclinical infection.

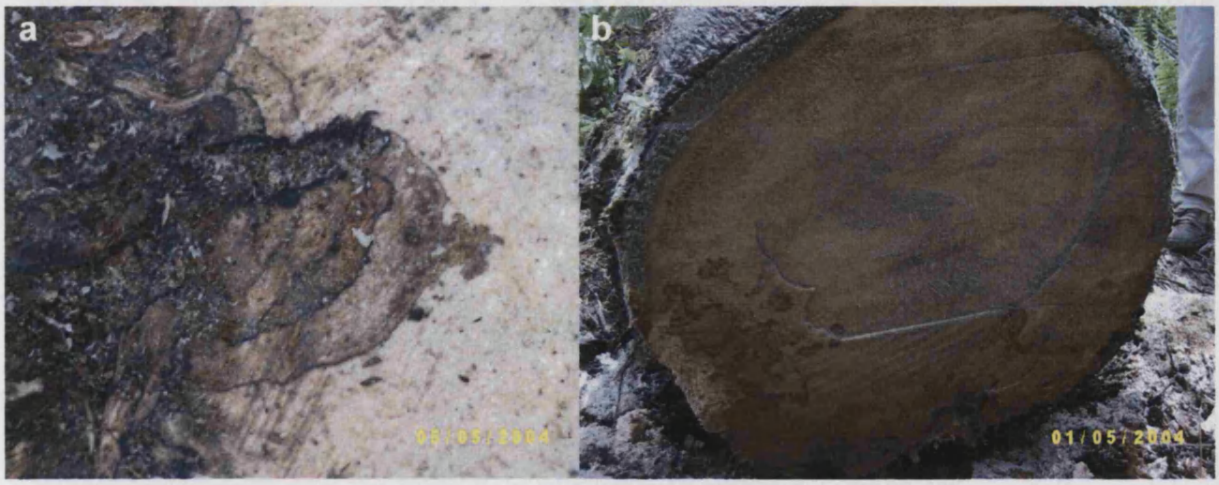

FIG. 18. Progression of infection from the root into the bole and transverse section of a heavily infected palm. **a** Severely infected root almost completely degraded and progress into the basal tissue. Infection is seen extending in waves delimited by dark brown pseudo-sclerotia. **b** Lesions have coalesced to cover much of the basal tissue. In this case it appears that an inoculum source has led to infection of roots on one side of the palm resulting in initial rot of only one section of the bole.

2.3.8 Spore Germination on Cut Frond Bases

Basidiospores germination was assessed on cut frond surfaces and within frond xylem vessels. This exposed tissue represents the most extensive and frequent wound sites created in plantations. After pruning, negative tension in severed xylem would result

in any liquid and particles being withdrawn deep into the corresponding vessel. Basidiospores were collected from two basidiophores on spatially separated, infected trees within a mature oil palm block. The spores were suspended in SDW (pH 5.0) to form a $1x10^8$ spores/ml suspension and were applied to fronds within 1 h. To observe germination from spores within submerged xylem vessels longitudinal sections were made; TS sections showing basidiospores were from the exposed wound surface. Eosin dye (2 mg/ml) was applied to the spore mixture to aid production of longitudinal sections as the red dye allowed visualisation of cuts along xylem vessels. A clear red line running longitudinally across the surface indicated successful xylem sections. Spores from two spatially separated (different infected trees) basidiophores were used to make the spore suspension applied to cut fronds. Dikaryotic mycelium was previously shown to be required to induce infection of oil palm seedlings (2.3.1) and to encourage anastomosis and possible infection; basidiospores from different basidiophores were applied concurrently to the cut fronds.

Fronds were cut by machete to leave a smooth, horizontal surface onto which 5 ml of the spore suspension was immediately applied. Cut frond bases were then covered with a plastic bag to prevent removal of spores by rainfall and to maintain high RH. After 2 and 4 days, treated fronds were sectioned and fixed for analysis using cryo-scanning electron microscopy. Five trees of the same age and from the same plantation were used and one frond from each tree was assessed for each day of the trial. To assess viability of the spores *in vitro* germination was assessed with spore suspensions diluted to $1x10^6$ spores/ml. One millilitre of this suspension was pipetted onto pH 5.5 water agar petri-dishes and left to germinate for 48 and 72 h.

2.3.8.1 Spore Germination October 2004

Spores germinated readily on water agar (pH 5.5) with germination rates ranging from 45-75% (Fig. 19). Statistical analysis showed that germination was not significantly greater after 72 h than 48 h, there was no significant difference between treatments and germination was not affected by application of eosin (Fig. 19). Therefore, a) spores were similarly viable from each bracket, b) mixing spores did not increase or decrease germination, c) germination from viable spores occurred within the first 48 h and d) addition of eosin did not impede germination. *In vitro* germination therefore

demonstrated that spores used to inoculate palms were viable at the time of inoculation.

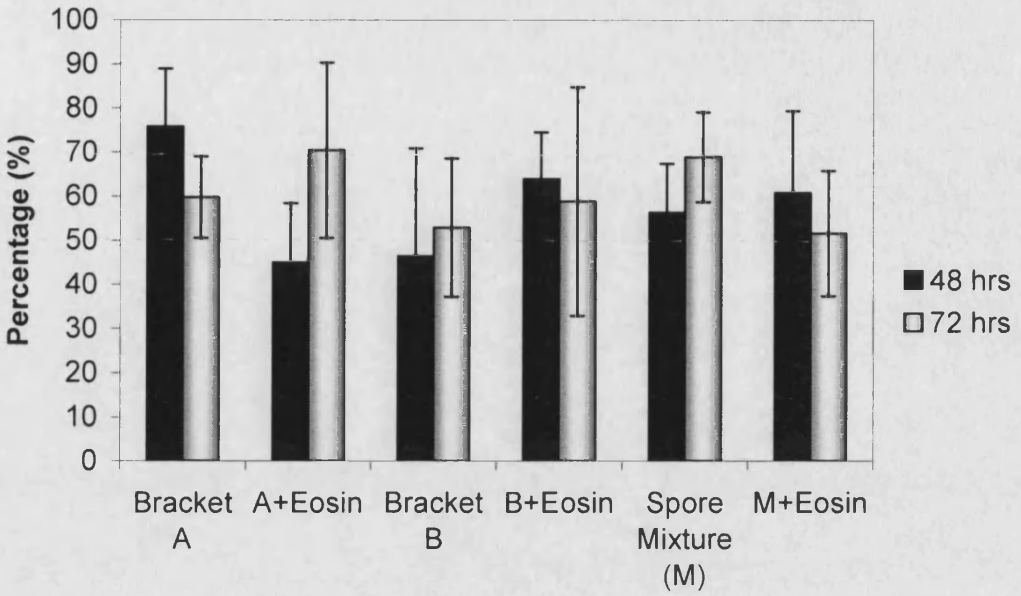

FIG 19. Germination of basidiospores on water agar pH 5.5 October 2004. Figure shows germination of treatments of 45-75%. There was no significant difference between germination rates after 48 and 72 h ($P < 0.6685$, df = 1) using one-way ANOVA, therefore most basidiospores, if viable, germinated within 48 h. There was no significant difference between spore germination in presence or absence of eosin (one-way ANOVA, $P < 0.767$, df = 1). No statistical differences were observed from germination between treatments by one-way ANOVA ($P < 0.2313$, df = 5). Comparison of means by Tukey-Kramer HSD showed no statistical differences between treatments ($P = 0.05$). Error bars represent standard deviation of means from germination on three replicate plates.

After collecting, sectioning and fixing inoculated fronds, samples were transported to the UK and viewed under SEM. Images of basidiospores from the exposed surface of the cut fronds are shown in Fig. 20a-d. Images of basidiospores from within xylem vessels are shown in Fig. 21a-d. Most images are from sections obtained 48 h after inoculation, samples after 72 h were heavily colonised by bacteria and contaminating fungal mycelia (Fig. 20c).

No evidence of basidiospore germination was observed from any of the cut fronds after either 48 h or 72 h. Many host cells were filled with *Ganoderma* basidiospores, but germ tubes were absent (Figs 20a, b & c). Fig. 20b shows higher magnification of basidiospores resting in host cell and absence of germination. After 72 h cut frond sections were heavily colonised by bacteria (Fig. 20c). Enhanced magnification

shows more clearly the ovoid basidiospores with a truncated apex, and absence of germination (Fig. 20d).

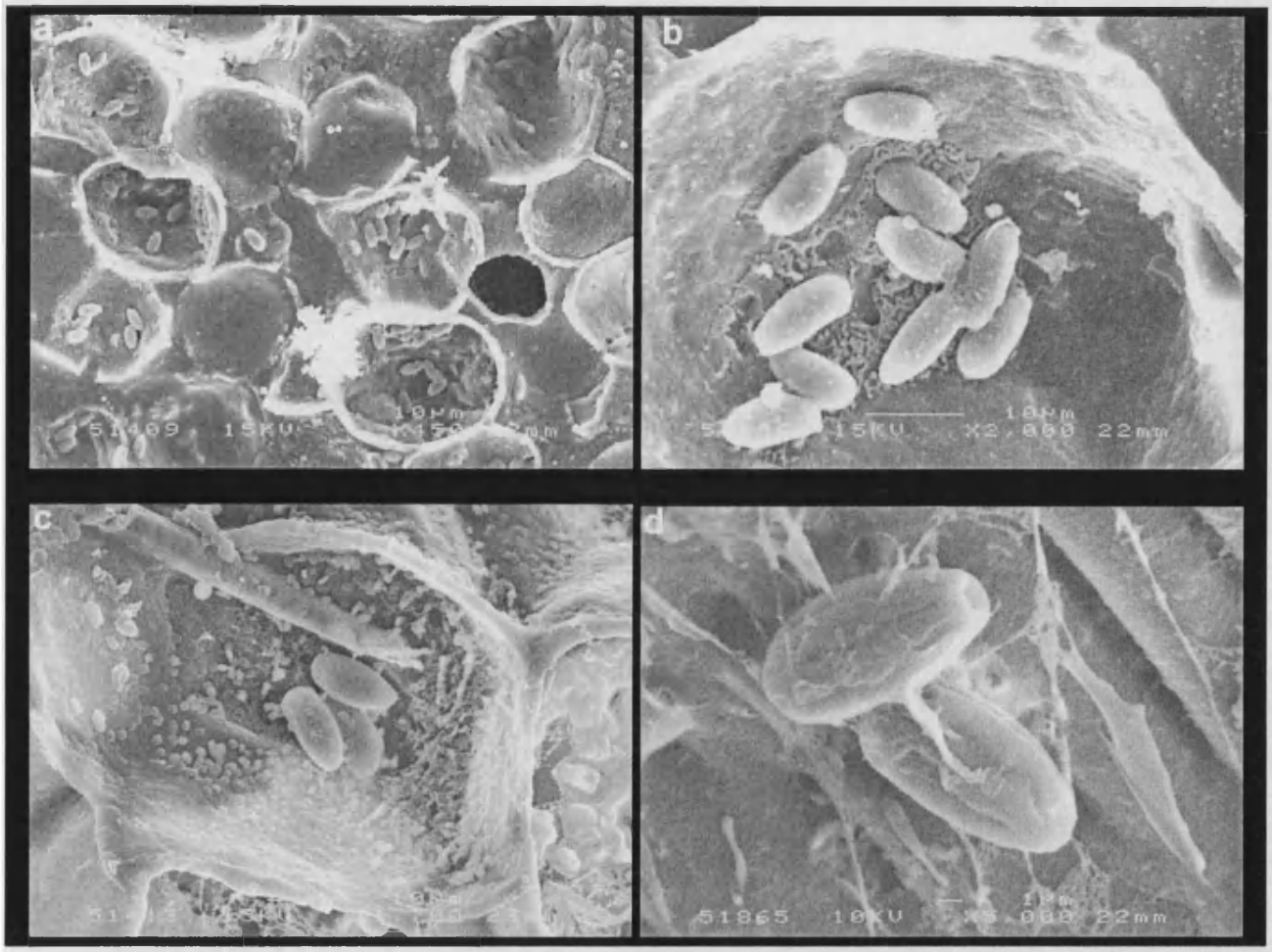

FIG. 20a-d. ***G. boninense*** **basidiospores on cut fronds October 2004.** Note absence of germination from basidiospores. Images a, b and d show basidiospores from 48h post inoculation and c shows basidiospores from 72h post inoculation.

Longitudinal sections cut along xylem vessels showed that basidiospores could be pulled inside the vessels. However, basidiospores were not observed more than 0.5cm into the vessel. Spores pulled into xylem vessels would not be exposed to UV and may not be subjected to the host wound responses, which may prove advantageous for germination. Yet images from within xylem vessels also show a complete absence of germination. Fig. 21a shows numerous *G. boninense* spores outside of and descending into a xylem vessel. Neither the spores outside, or within the xylem vessel show any signs of germination. Fig. 21b shows higher

magnification of those cells within the vessel and confirms absence of germ tubes. Fig. 21c, d shows longitudinal sections of xylem vessels and absence of germination.

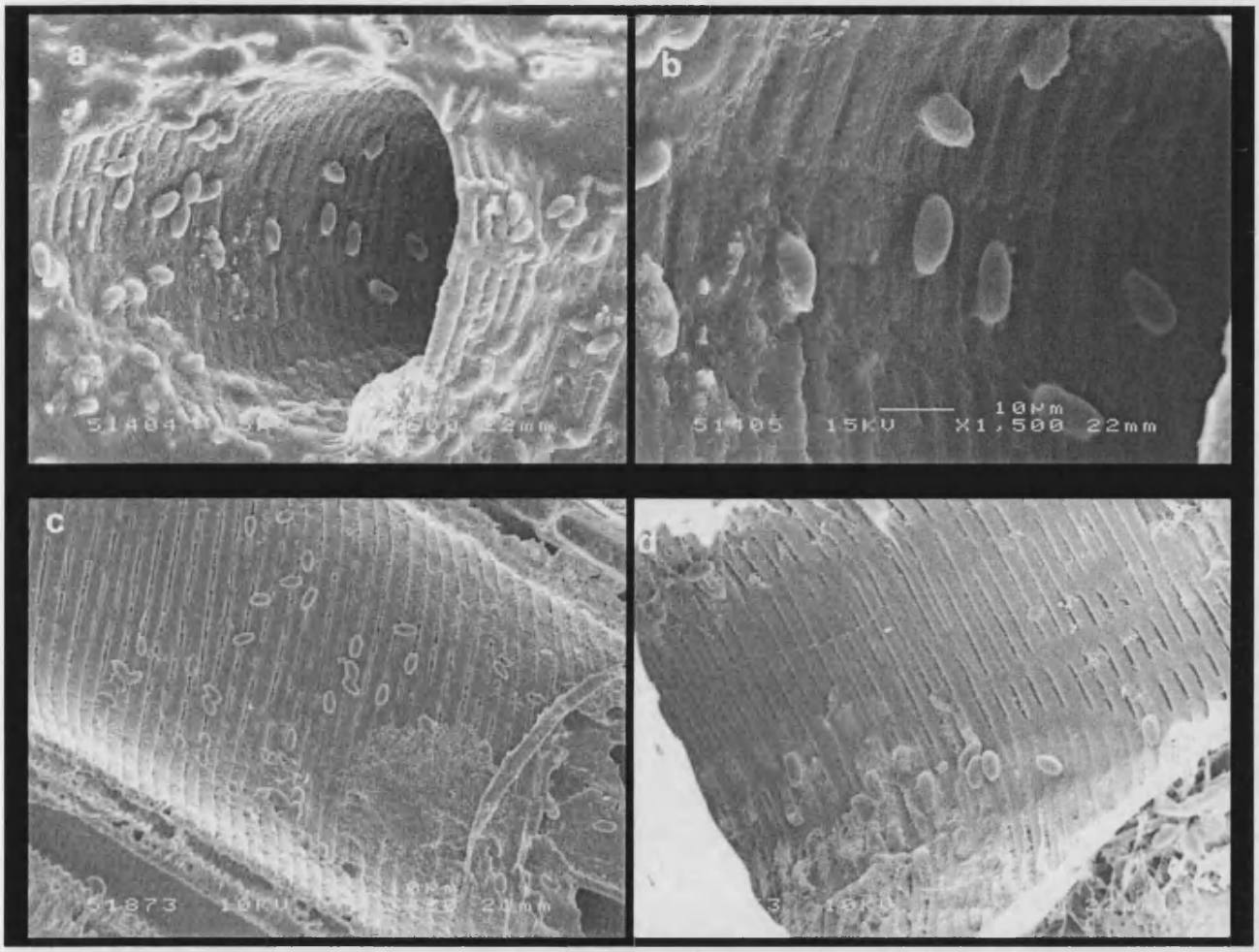

FIG. 21a-d. ***G. boninense*** **basidiospores in xylem vessels on cut fronds October 2004.** Note absence of germination. Images are from 48hrs post inoculation.

2.3.8.2 Spore Germination May 2005

Because of the unexpected failure of spores to germinate on cut fronds, it was decided to repeat the experiment but with a number of variations. Basidiospores were collected and spore suspensions prepared as before, with *in vitro* viability assessed. Basidiospores were inoculated not only onto cut fronds, but also onto cut peduncles and wounded trunks of mature trees. This was to determine if basidiospores germination was inhibited on some but not all tissue types above the others for germination. Also, basidiospores were applied to cut surfaces immediately after

wounding (3 reps for each treatment) and also to 2 wk old wounded surfaces (3 reps for each treatment).

Basidiospores again germinated readily under laboratory conditions and there was no significant difference between treatments (Fig. 22). The lowest average germination was 57% and the highest was 85% after mixing spores from the two basidiophores (Fig. 22). Eosin was added to all basidiospore suspensions, as it was previously shown not to affect germination of basidiospores (Fig. 19) in the laboratory and addition was necessary to visualise xylem vessels on cut fronds and peduncles when sectioning.

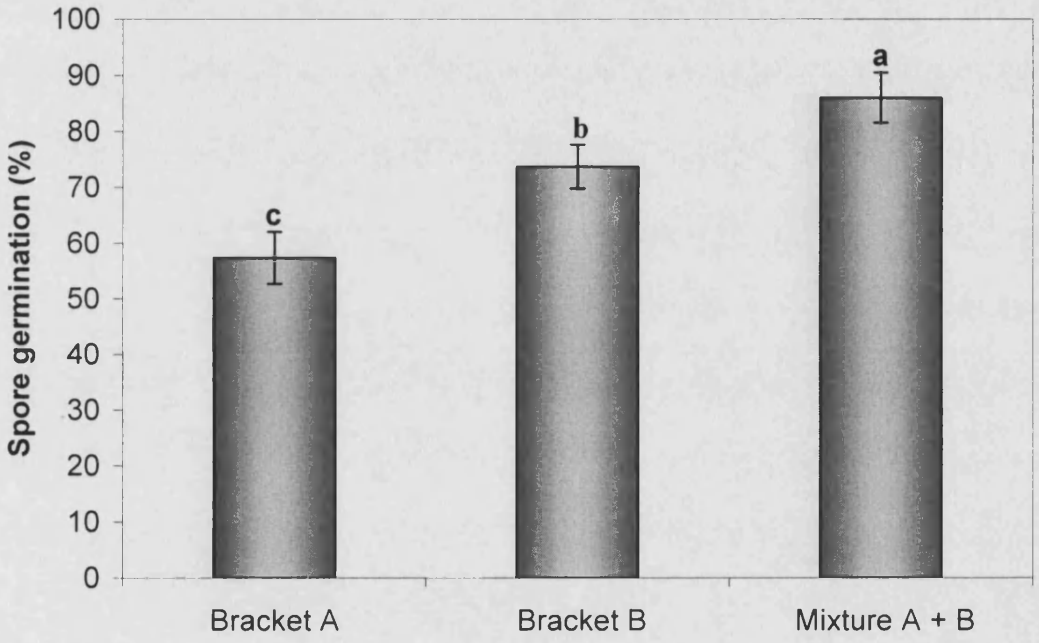

FIG. 22. Germination of basidiospores from two basidiophores, independent and combined. Statistical differences were observed between spore germination in water alone or after addition of eosin dye by one-way ANOVA ($P < 0.0005$, df = 2). Comparison of means by Tukey-Kramer HSD showed no statistical differences between treatments ($P = 0.05$). Error bars represent standard deviation of means from germination on three replicate plates. Germination was assessed on water agar pH 5.5 + eosin May 2005.

Basidiospores germinated readily on wounded surfaces in the field (Fig. 23). Germination was easier to observe on wounded surfaces inoculated immediately compared to surfaces left for 2 wks due to build-up of contaminants. All wounded surfaces, peduncles, trunk surfaces and fronds supported high germination. This contrasts markedly with previous results where spores were unable to germinate on

cut fronds. Different basidiophores were used in May and these spores were more viable *in vitro* than those collected previously. Enhanced germination *in vivo* may be attributed to this, however climatic factors cannot be ruled out at this stage. There was no observable difference between germination within xylem vessels or on the exposed surface; germination occurred wherever spores came to rest. Wounded trunk tissue and peduncles showed markedly more contamination than frond tissue. Peduncle tissue is less fibrous and degrades rapidly in the field; in view of its function in supplying fruit bunches the tissue undoubtedly contains many sugars and other nutrients that encourage colonisation by saprophytic fungi and bacteria. Trunk tissue is also less fibrous than fronds and is often seen covered in a green spore forming fungus when wounded.

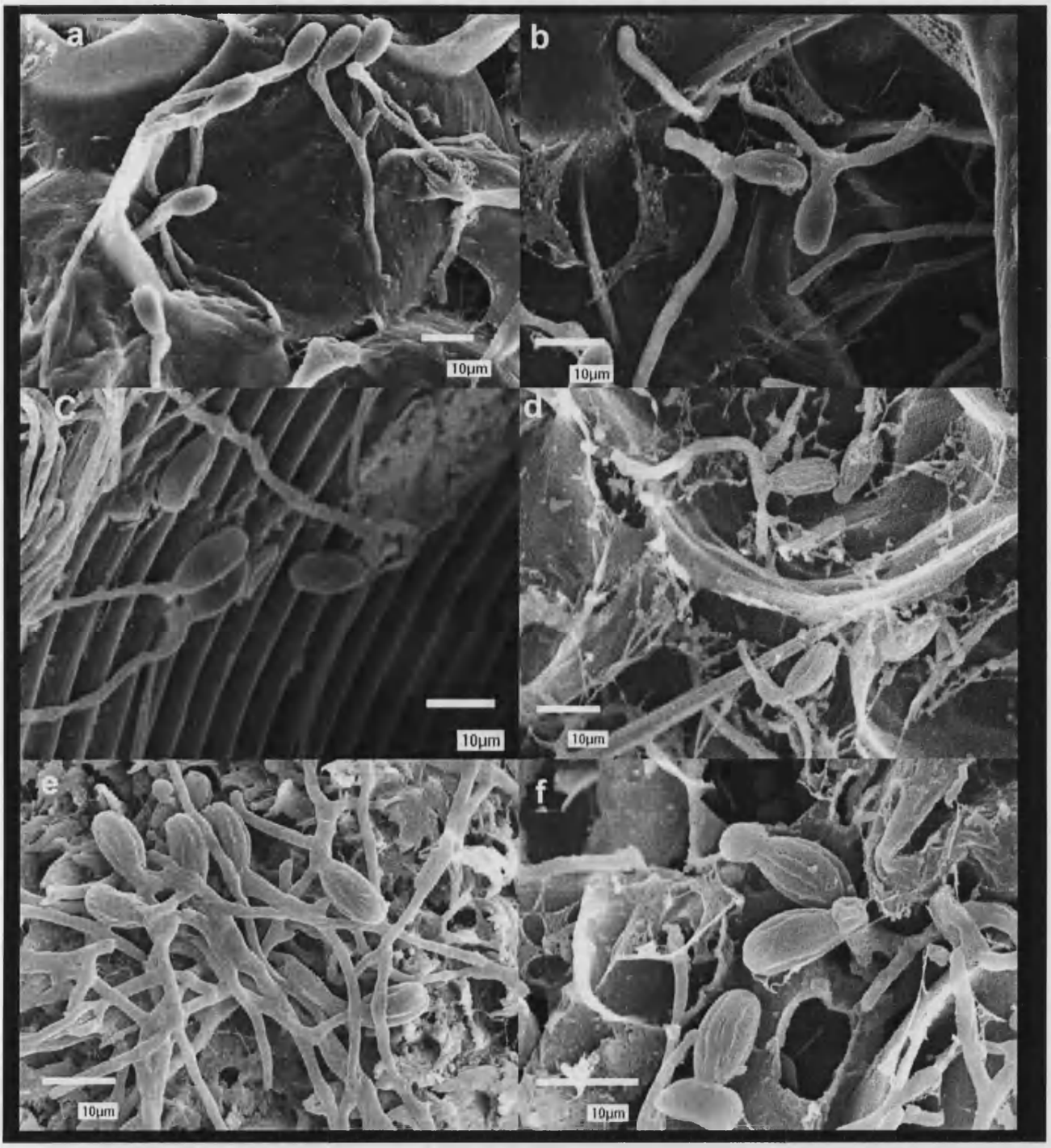

FIG. 23a-d. **Germination of *G. boninense* basidiospores on wounded fronds, peduncles and trunk tissue.** **a & b** Germination of *Ganoderma* basidiospores in cut frond (petioles) parenchyma cells. **c** Basidiospore germination in xylem of cut frond. **d** Spore germination on cut fruit bunch stalks (peduncles). **e** Mass of germinating spores with germinating hyphae in very close association on wounded trunk surface. **f** Initial stages of basidiospore germination on wounded trunk surface. All pictures show spores 48 h post inoculation to wounded surface.

2.3.9 Vessel Lengths in Oil Palm

To determine how far a basidiospore may be drawn into a severed xylem vessel, fluorescent particles, eosin dye and SEM was used to provide an estimate of xylem vessel lengths. Addition of eosin dye to a wounded frond surface allows visualisation of xylem vessels (Fig. 25). The dye can be observed progressing over 20 cm into the tissue and is not limited by the presence of end walls. End walls serve to stop the progression of microbes within xylem tissue and consist of secondary wall permeated by pits where only primary wall has been laid down (Fig. 24). A longitudinal section from a cut frond surface showed the presence of an end wall approximately 3 mm from the cut surface. This vessel may have been much longer and to determine how long xylem vessels are, and therefore how far a basidiospore may be pulled into wounded fronds due to negative pressure, small fluorescent particles were mixed with eosin and applied to cut surfaces. Analysis of sections using UV microscopy was carried out to detect presence of the red particles deep within the vascular system (Fig. 25). Red particles were observed within tissue to a depth of 10 cm and frequency of particles was greatest nearest the cut surface. In tissue sections beyond 10 cm no red particles could be observed. This suggests that vessel lengths do not extend far beyond 10 cm and only few extend to these lengths. *Ganoderma* basidiospores could conceivably be dragged a substantial distance within oil palm tissue and therefore be shielded from competition, damaging UV radiation, extreme temperature and may gain considerable access to frond bases close to the trunk.

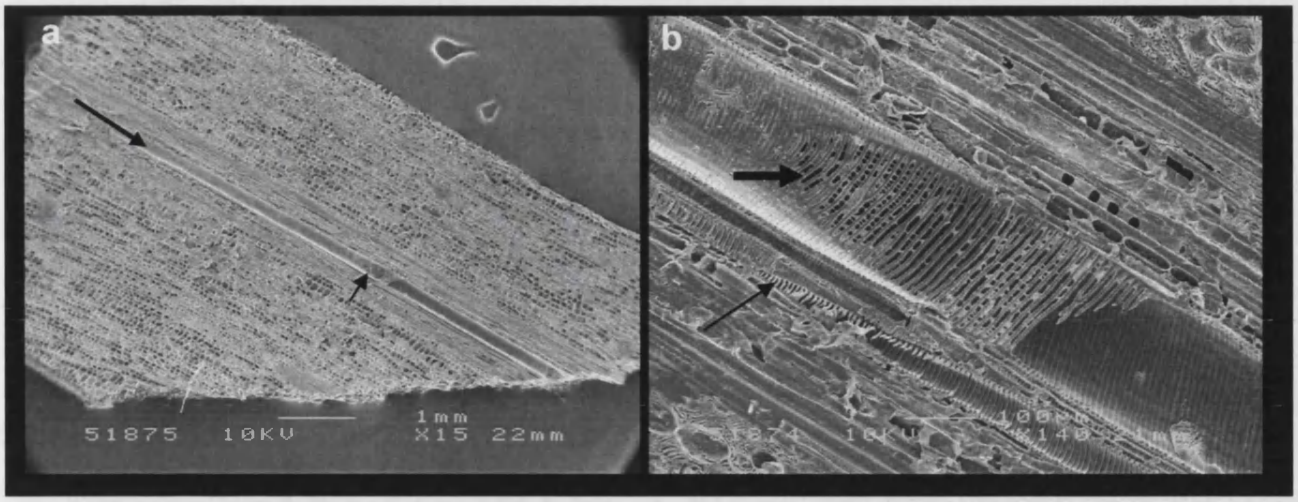

FIG. 24. Xylem vessel end walls in fronds. **a** longitudinal section from cut surface (large arrow) showing an exposed xylem vessel and end wall (small arrow). **b** higher magnification of end walls from a wide xylem vessel, and a narrower adjacent vessel, showing a pit field as it tapers to an end wall (large arrow); the adjacent narrow vessel also shows a vessel terminating (small arrow).

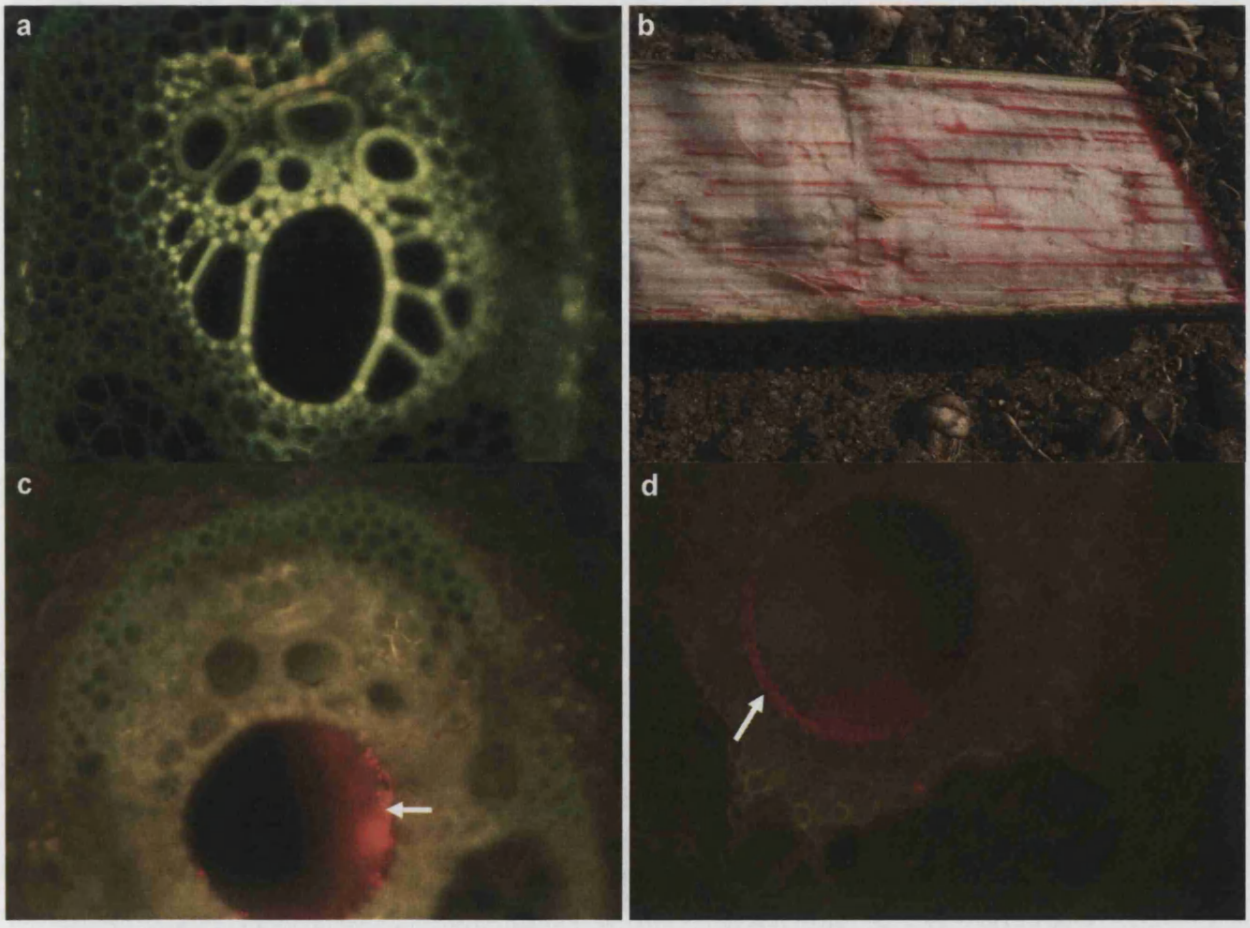

FIG. 25. Vascular anatomy and length recorded by fluorescent particles and eosin dye. **a** Section from an untreated oil palm frond showing absence of fluorescent particles. **b** Longitudinal section showing xylem vessels stained with eosin. **c** Red particles (arrow) within a xylem vessel 5 cm below a cut surface. **d** Red particles (arrow) visualised at a depth of 10 cm.

2.3.10 Quantification of Airborne Spores Within Plantations

If basidiospores are important in disease aetiology, the quantity of and variation in basidiospore production within plantations needed monitoring. However, accurate quantification of basidiospores within plantations and circadian influence on spore release has never been determined. To rectify this, spore samples were taken in the early morning, midday, early evening and midnight over a 4 day period and average spore concentration/m^3 determined as described above using a Biotester®.

Spore numbers were lowest at 07.00 h (Fig. 26), with a mean of *ca.* 2,000/m^3. This increased to *ca.* 4,000 spores/M^3 at 12.00 h and reached a peak of *ca.* 11,000 spores/m^3 at 19.00 h, after which point spore concentration dropped markedly to *ca.* 4,500 spores/m^3 at 24.00 h. This indicates that most spore release occurs during daylight hours.

Atmospheric spore levels are variable depending on the inoculum source available to *G. boninense*. Air samples were taken from eight and seventeen year-old plantings and from a three year-old windrow at midday (Fig. 27). Infection levels in seventeen-year old trees was considerably more than in eight year-old plots, therefore more basidiophores are likely to be present producing spores in mature stands. Observed basidiospore levels in 17 year-old plots of *ca.* 4,500 spores/m^3 compared to *ca.* 3,000 spores/m^3 in 8 year-old plantings confirm this. Considerably fewer basidiospores (*ca.* 2,000 spores/m^3) were observed in 3 year-old windrows. Unfortunately no 1 year-old windrows were available for sampling at Bah Lias during testing; it is likely that considerably more basidiospores are produced from fresh windrows because of the large amount of material available for *Ganoderma* colonisation. Hasan and Turner found that palm trunks were infective for approximately 2 years (99), after this time trunks are mostly decayed with few productive basidiophores. Further testing in windrows is advisable to determine the danger posed by spore production from these sources. However, spore production is likely to be more affected by abiotic factors since windrows are exposed to greater stress factors such as UV radiation than basidiophores in mature plantings.

Samples were also collected from a fresh bracket with a pore surface of approximately 10 cm^2 from 07.00 h to 23.00 h and produced on average *ca.* 140,000 spores/min during daylight hours. Further sampling would be required during hours of darkness in the early morning to provide a firm estimate of spore production/day.

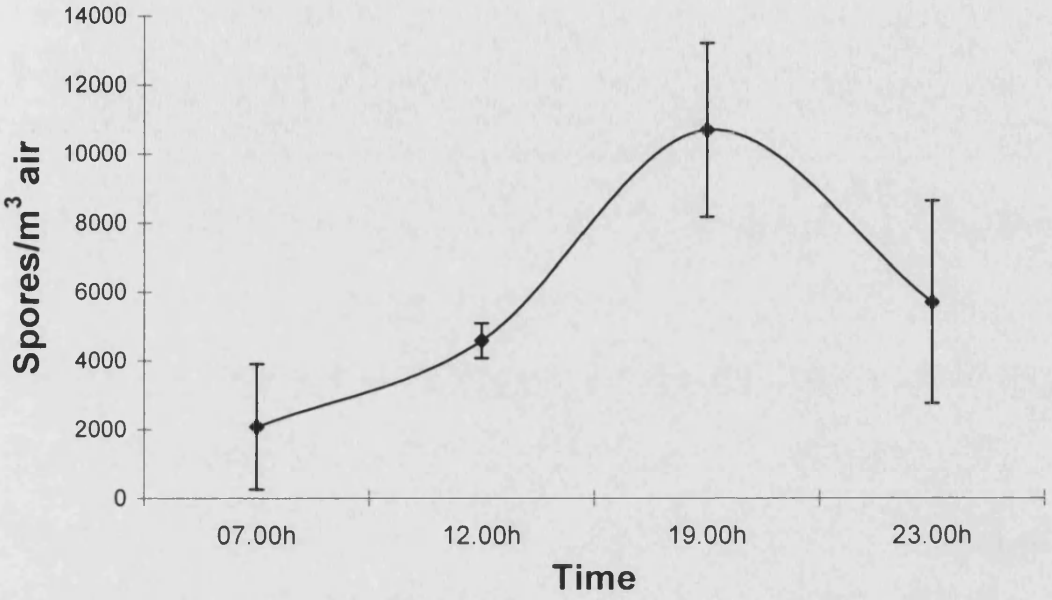

FIG. 26. Dimensional fluctuation of aerial basidiospore numbers in a 17-year old plot. Error bars represent standard deviation of the mean of four samples at each time point.

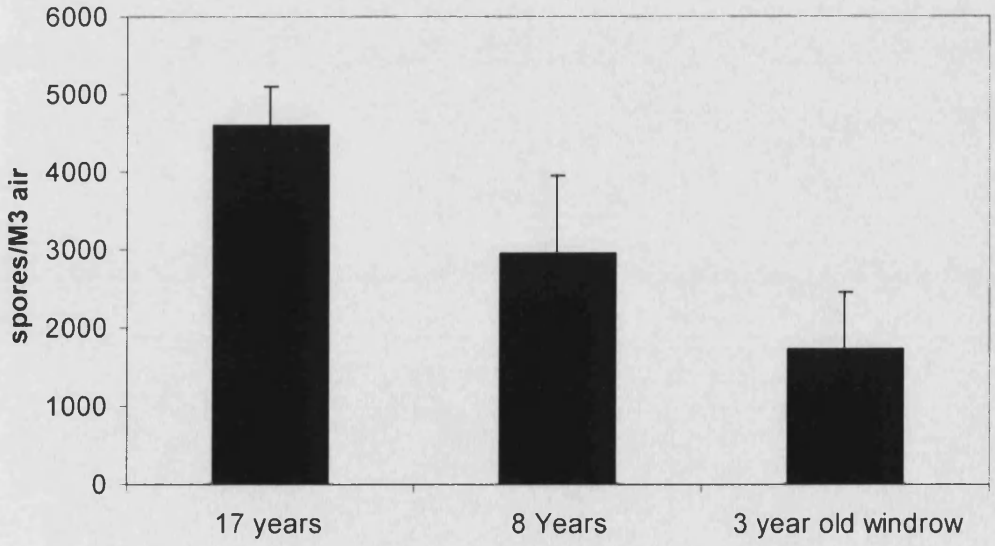

FIG. 27. Variation in aerial basidiospore numbers at different locations. Error bars indicate standard deviation of mean from three samples per location. There was no significant difference in atmospheric spore concentration between the sample areas by one-way ANOVA ($P < 0.239$, df = 2).

2.3.11 Growth of *G. boninense* in Field Soil and Organic Debris

G. boninense has been reported to be soil-borne by researchers investigating control of BSR (217). It has also been suggested that basidiospores can germinate and colonise debris trapped in frond axils (frond debris (FD)) before subsequently invading the palm stem causing USR (78). In order to test these suggestions *in vitro* growth of *Ganoderma* was attempted in soil and FD collected from Bah Lias research station North Sumatra, Indonesia. Half of the material was autoclaved for 1 h at 121°C before dispensing into sterile containers. *G. boninense* grown on sterile wheat grains was then used as inoculum to provide a sufficient food base. Non-autoclaved material was placed directly into sterile containers and inoculated with *Ganoderma*-colonised wheat grain. To ensure that aeration was not a growth-limiting factor, tubes were randomly inoculated either at the top of the tube near the cap and the others inoculated at the bottom. In addition, cut oil palm roots were placed in the centre of half of the containers (randomly selected) of each treatment (autoclaved material/non-autoclaved) to determine if root exudates had any effect on growth. Five replicate tubes were made for each treatment and radial growth was assessed every second day over an eight-day period.

When material was not sterilised, soil and FD were unable to support growth of *G. boninense*. Growth in sterilised FD was rapid and could be visualised as mycelial cords emanating from the inoculum source (Fig. 28). Hyphae had progressed approximately 30 mm from the inoculum source in sterilised FD within 8 days (Fig. 29). Growth in soil was slower and reached approximately 15 mm after 8 days. The soil contained less organic material than FD and therefore reduced nutrient availability probably accounted for less radial growth. Oxygenation did not appear to be limiting, as growth was equivalent regardless of inoculum placement at the bottom or near the cap within growth chambers.

Freshly cut seedling roots were added to some of the containers to determine if root exudates could stimulate chemotropic growth of *G. boninense*. However, the fungus did not grow in any of the sterilised soil chambers supplemented with roots. Sterilised FD supplemented with roots supported good growth in initial stages and had

reached 15 mm after 6 days. However, as mycelium approached the roots, growth was halted and little more occurred.

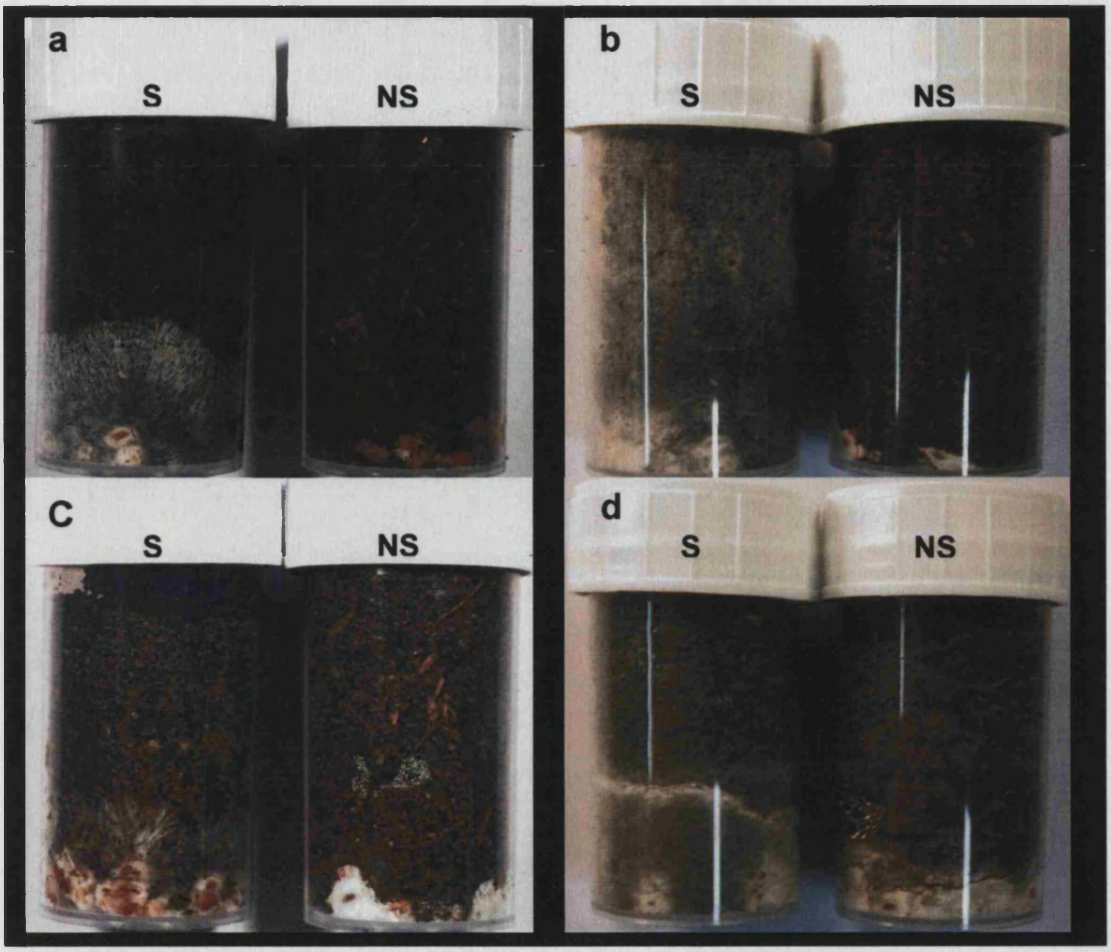

FIG. 28. Hyphal extension of *G. boninense* in sterile and non-sterile soil and frond debris (FD). NS = non-sterile, S = sterilised by autoclaving. Colonised wheat grains were used as inoculum source. **a** Mycelial growth in FD after 4 days. **b** Extension in FD after 10 days. **c** Growth in soil after 4 days. **d** Hyphal extension in soil after 10 days.

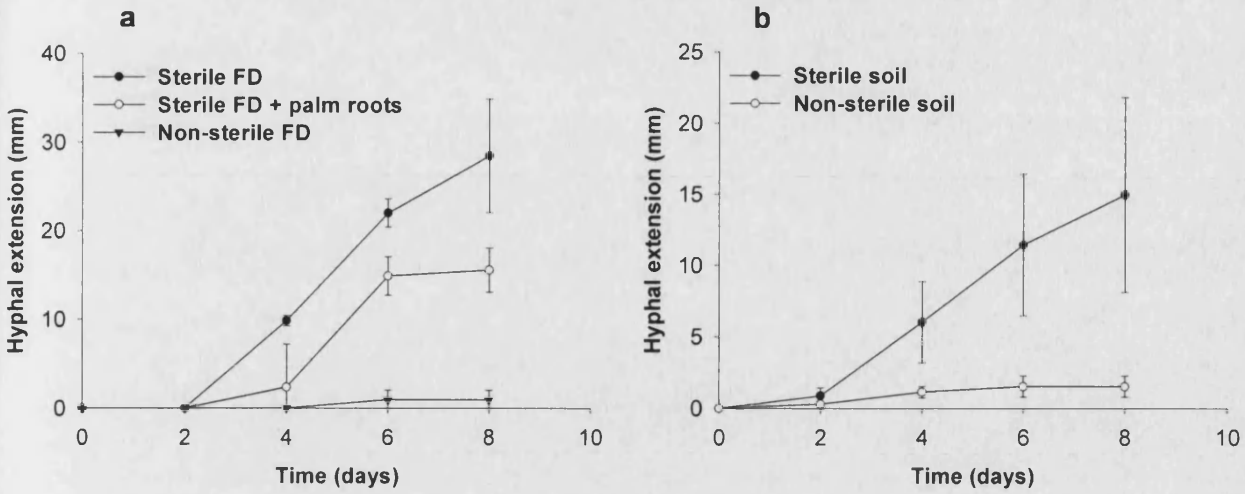

FIG. 29. Hyphal extension of *G. boninense* within sterile and non-sterile frond debris and plantation soil from North Sumatra, Indonesia and the influence of added palm roots. FD = frond debris. **a** Three hyphal extension measurements were made from each growth chamber per time point and error bars represent standard deviation of means from 5 replicates (growth chamber). Bivariate ANOVA showed that there was a significant difference between hyphal extension from different treatments ($P < 0.0001$, df = 51) and comparison of means by student's t-test showed that hyphal extension was significantly higher in sterile FD compared to FD + roots and the lowest hyphal extension occurred in non-sterile conditions ($P = 0.05$). **b** More soil was collected and therefore a total of 20 replicates were used for sampling in this case. Bivariate ANOVA showed that there was a significant difference between hyphal extension from different treatments ($P < 0.0001$, df = 159) and comparison of means by student's t-test showed that hyphal extension was significantly higher than in sterile soil than in non-sterile conditions ($P = 0.05$).

2.3.12 Genotyping Isolates of *G. boninense*

Root infection by *G. boninense in vitro* has been well established but genetic studies have shown high genetic variability within plantings and even between neighbouring palms (143, 173), which questions the role of vegetative spread in infection. Mitochondrial DNA RFLPs was conducted in Malaysia but sampling was not conducted in either USR infected trees or fallen palms (FP) (143). Therefore population structures within BSR and USR infected palms have not been compared. Furthermore, the possible importance of FPs as inoculum sources for disease has so far been overlooked; these become heavily colonised by *Ganoderma* and may be important inoculum sources in plantations (Fig. 30). Using RAMS aims to investigate population structures within plantings, between BSR and USR infected palms and to determine if a relationship can be found between BSR infections and FPs.

Isolates were collected from five plantings in two Lonsum Estates: Bah Lias and Sungei Bejanker. Plots were given a number code; the first two numbers are the year

of planting and the three subsequent numbers represent the number of trees in the planting. Plots 86-200, 85-200 and 88-300 were from Bah Lias and 84-300 and 86-400 were from Sungei Bejanker (Appendix, Figs 1-5). Mature plots were chosen so that incidence of BSR would not be limiting and that preferably fallen palms would be located adjacent to standing BSR infections. One BSR palm was felled in each plot and isolation was attempted from rotting tissue and basidiophores using GSM. Sampling was also attempted from tissue and basidiophores from at least one adjacent fallen palm in each plot and from basidiophores on any nearby BSR infected palms. These palms were not felled because of financial considerations and therefore sampling from rotting tissue was not possible. One USR tree was also felled from each plot with sampling from tissue and basidiophores. Success of isolation from palm tissue was not high and was particularly low from fallen palms, but was most easily facilitated from basidiophores. Isolates were then subcultured onto PDA before extraction of DNA, sequencing and fingerprinting as described above. Isolates used for sequencing and fingerprinting (57 isolates) are listed in Table 1.

Plot	*G. boninense* isolate	Plot	*G. boninense* isolate
84:300	**USR R1**	**86-400**	**USR R6**
SBJ	USR R2	**SBJ**	USR R4 S2
	USR R5		BSR B1 S2
	USR R6		BSR B2 S2
	USR R8		BSR B3 S2
	USR R2 S2		BSR B5 S2
	FPA B1 S2		BSR BB R4
	FPA B2 S2		BSR BB R5
	FPA B3 S2		BSR BB B1 S2
	FPC R2 S2		BSR BB B3 S2
	FPC R3 S2		BSR BC B1 S2
	FPC R4 S2		BSR BC B3 S2
	FPC B1 S2		BSR BC B5 S2
85-200	**USR R2**		BSR BD B3 S2
BLRS	USR R3	**86-200**	**USR R1**
	USR R2 S2	**BLRS**	USR R3
	BSR R3		USR R4
	BSR R6		USR R9
	BSR R12		BSR B1 S2
	BSR B1 S2		BSR BD B1 S2
	BSR B2 S2		BSR BD B3 S2
	BSR B3 S2		BSR BD B4 S2
	BSR B5 S2	**88-300**	**BSR R9**
	FPA R1	**BLRS**	BSR R1 S2
	FPA R3		FPA R9
	FPA B3 S2		FPA R11
	FPA B5 S2		
	FPA B7 S2		
	BSR BB B3		
	BSR BB B4		
	BSR BB B5		

Table. 1. ***G. boninense*** **isolates from two estates in North Sumatra.** Plots 86-200, 85-200 and 88-300 were from Bah Lias and 84-300 and 86-400 were from Sungei Bejanker. Plots are separated with thick lines. All isolates from the same tree are grouped together and have the same prefix. FP = fallen palms, suffix A,B,C denotes different fallen palms. BSR = basal stem rot infected palm. USR = upper stem rot infected palms.

FIG. 30. Fallen palm from plot 88-300 in Bah Lias estate, North Sumatra. Basidiophores can be observed along the length of the palm trunk and some basidiophores are shown within circles.

2.3.12.1 Sequencing of *Ganoderma* ITS1, 5.8S rDNA and ITS2

Sequencing confirmed the identity of most of the isolates as *G. boninense* after BLAST analysis using the NCBI database. Previous sampling of *G. boninense* in from oil palm by Utomo *et al* (235) and numerous species of the *G. lucidum* complex by Moncalvo *et al* (146, 147) provided many sequences for comparison. All isolates obtained from BSR and USR infected standing palms were found to be *G. boninense*, however several isolates from FPs appeared to be different *Ganoderma* species based on BLAST comparisons with sequences from the NCBI genetic sequence database. For example, isolate FPA B1 S2 from plot 85-200 had most homology to *Ganoderma fornicatum* isolates from Taiwan and FPC R4 S2 was most closely related to *Ganoderma gibbosum* from mainland China. Thus, in this study, only *G. boninense* was found to cause infection of oil palm whilst other *Ganoderma* species are competitive saprophytes of fallen palm tissue. Complete identity of the 5.8S rDNA was observed in all isolates from Sumatra and also with *G. boninense* isolates from oil palm in Indonesia (235). ITS1 and ITS2 are more variable than 5.8S rDNA and have

been used for numerous interspecific phylogeny studies (146, 209). Three residues in ITS1 and one residue in ITS2 showed variability, however this was not sufficient for determination between individuals (Fig 31). Inability to differentiate between closely related isolates was also observed by Latiffah *et al* (122) using RFLP of ITS1 and ITS2 on populations within oil palm and coconut. For greater discrimination, randomly amplified microsatellites (RAMS) was used to fingerprint isolates.

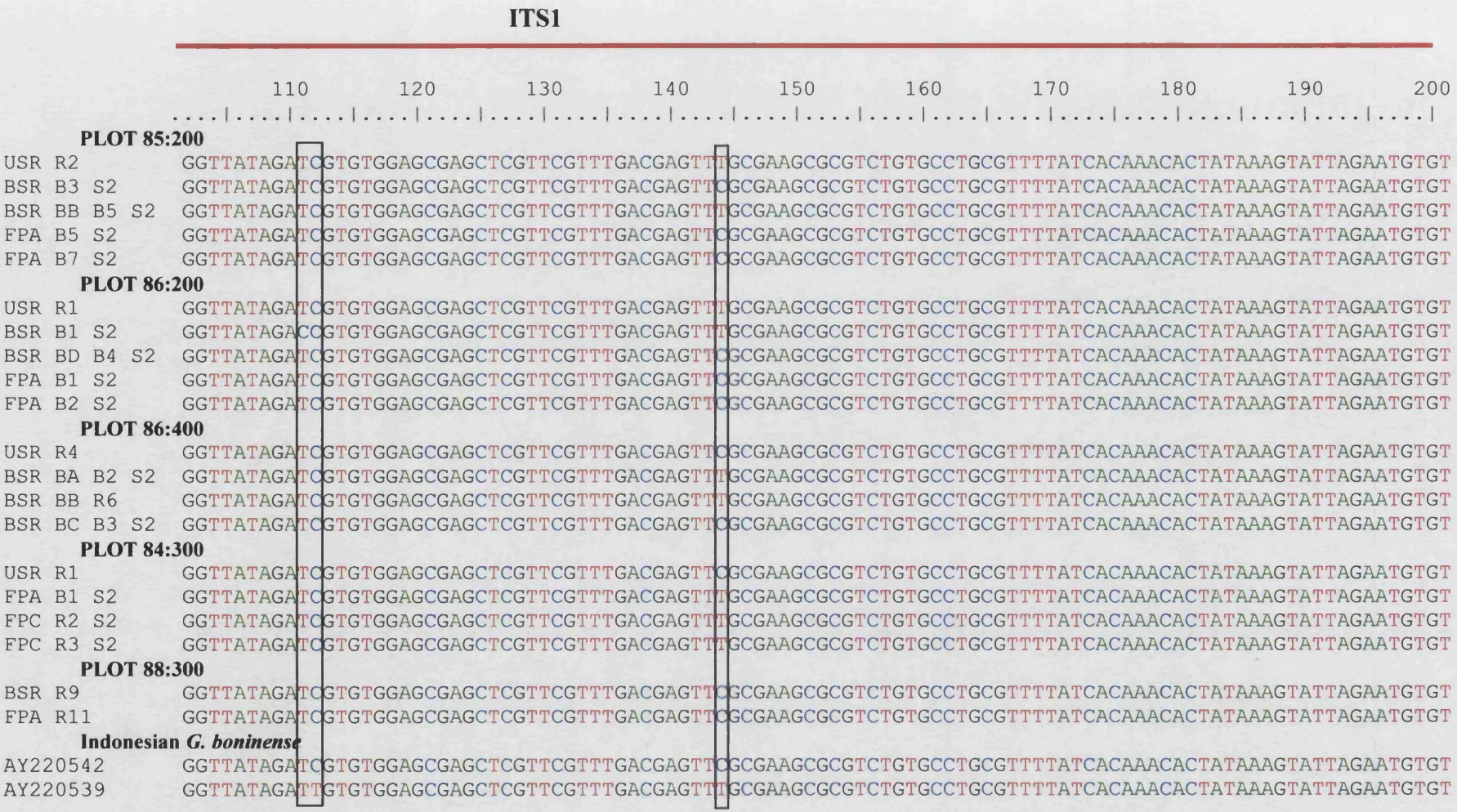

FIG. 31b. Genetic Sequence Alignment of *G. boninense* ITS1, 5.8s rDNA and ITS2. Selected isolates obtained from the 5 plots in Northern Sumatra (plots 85:200, 86:200, 86:400, 84:300 and 88:300) and two *G. boninense* sequences (obtained from the NCBI database) from isolates obtained from oil palm in Indonesia that show extremely high homology with isolates from this study. Variable residues are indicated by a box, all other resiudes are conserved throughout all isolates.

FIG. 31c. Genetic Sequence Alignment of *G. boninense* ITS1, 5.8s rDNA and ITS2. Selected isolates obtained from the 5 plots in Northern Sumatra (plots 85:200, 86:200, 86:400, 84:300 and 88:300) and two *G. boninense* sequences (obtained from the NCBI database) from isolates obtained from oil palm in Indonesia that show extremely high homology with isolates from this study. Variable residues are indicated by a box, all other resiudues are conserved throughout all isolates.

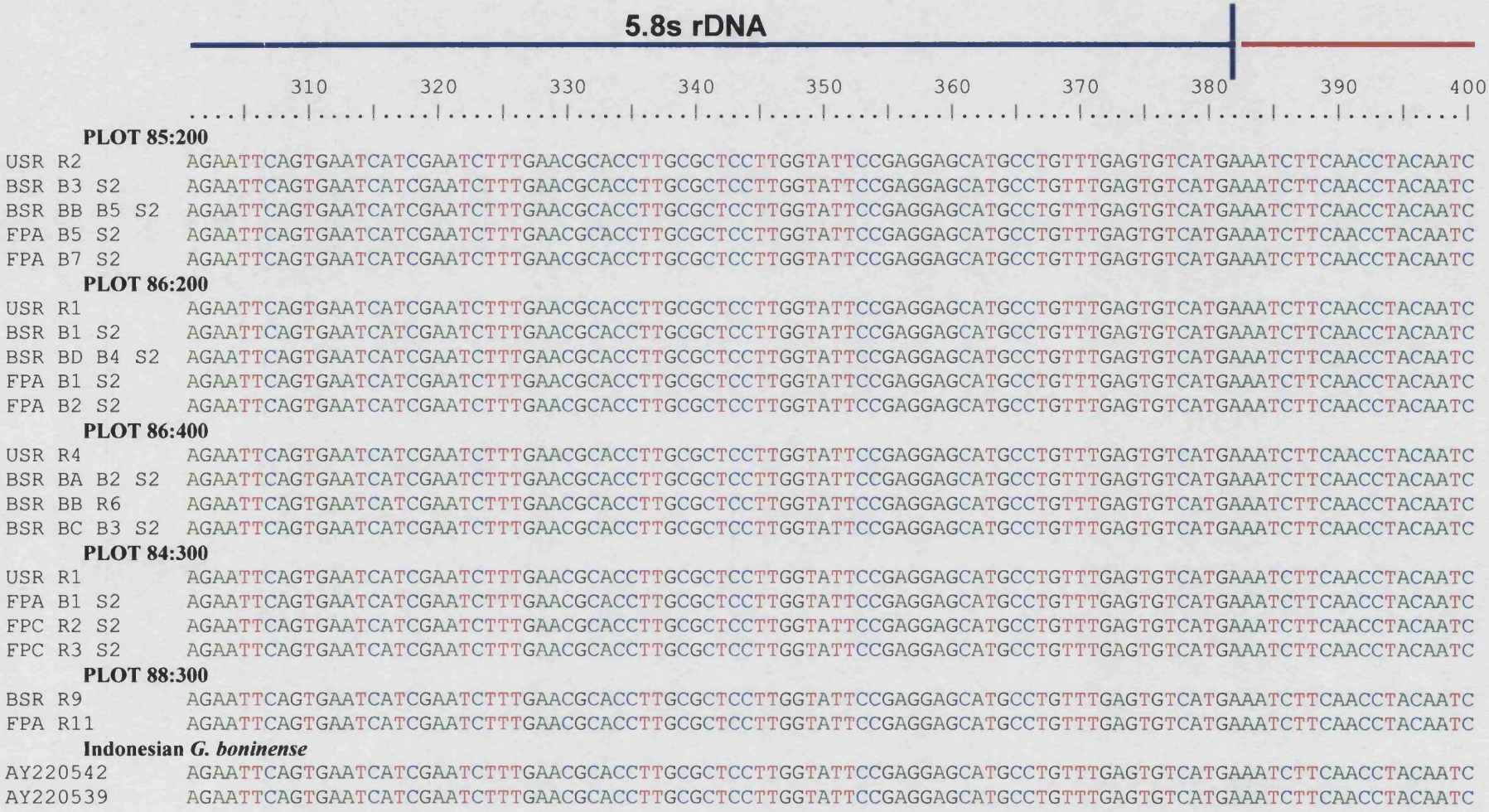

FIG. 31d. Genetic Sequence Alignment of *G. boninense* ITS1, 5.8s rDNA and ITS2. Selected isolates obtained from the 5 plots in Northern Sumatra (plots 85:200, 86:200, 86:400, 84:300 and 88:300) and two *G. boninense* sequences (obtained from the NCBI database) from isolates obtained from oil palm in Indonesia that show extremely high homology with isolates from this study. Variable residues are indicated by a box, all other resiudues are conserved throughout all isolates.

FIG. 31e. Genetic Sequence Alignment of *G. boninense* ITS1, 5.8s rDNA and ITS2. Selected isolates obtained from the 5 plots in Northern Sumatra (plots 85:200, 86:200, 86:400, 84:300 and 88:300) and two *G. boninense* sequences (obtained from the NCBI database) from isolates obtained from oil palm in Indonesia that show extremely high homology with isolates from this study. Variable residues are indicated by a box, all other resiudues are conserved throughout all isolates.

FIG. 31f. Genetic Sequence Alignment of *G. boninense* ITS1, 5.8s rDNA and ITS2. Selected isolates obtained from the 5 plots in Northern Sumatra (plots 85:200, 86:200, 86:400, 84:300 and 88:300) and two *G. boninense* sequences (obtained from the NCBI database) from isolates obtained from oil palm in Indonesia that show extremely high homology with isolates from this study. Variable residues are indicated by a box, all other resiudues are conserved throughout all isolates.

2.3.12.2 Randomly Amplified Microsatellites (RAMS)

Fingerprinting of isolates was conducted to address three key issues: i) Are neighbouring palms infected by the same isolate of *G. boninense*? ii) Is the same isolate of *G. boninense* found in adjacent fallen palms? iii) Are BSR and USR infections the result of single infection events and thus contain only one *G. boninense* genet?

The RAMS amplification adapted from Hantula *et al* (50) provided 6-12 clear bands per amplification, ranging from 400-1500 base pairs for each *G. boninense* isolate. Gel images were scored manually and identical banding patterns were regarded as the same clonal; a binary matrix was compiled from the different profiles and is shown in Table 2. This technique was reproducible and Fig. 32 shows PCR reactions from multiple isolates from a single BSR infected palm showing the same RAMS profile, indicating that isolates are clonal. The procedure was powerful enough to show up differences between isolates that were inseparable by ITS sequencing. Two isolates from a BSR infected tree show identical banding patterns, whereas isolates collected from an adjacent FP showed multiple heterogeneous bands that could be used to discriminate between individuals (Fig. 33).

Examination of the RAMS profiles did not reveal any identical genets from BSR infected trees and in adjacent FPs from any of the plots (Table. 2). Therefore there was no evidence to indicate vegetative spread of the disease from FPs to neighbouring palms. Only one plot contained adjacent BSR palms that appeared to have identical fingerprints. BSR BC B5 S2 had an identical band pattern to BSR BD B3 S2 in plot 86:400 from Sungei Bejanker (Fig. 34), in all other cases RAMS profiles from isolates obtained from separate trees were distinct. For two identical banding patterns to occur on adjacent palms, infection of the trees is almost certain to have occurred through vegetative mycelial growth. Recombination during meiosis leads to alterations in the genome that could alter RAMS profiles and the likelihood of two compatible basidiospores originating from the same individual is low since the tetrapolar mating system in *G. boninense* encourages outcrossing. Therefore, infection in this case is probably as a result of vegetative spread of the disease either by direct root-contact between trees or independent contact to an inoculum source in the field.

In all cases, isolates obtained from individual USR infections had identical band patters (Fig. 35). Banding patterns showed no homology between trees but the presence of only one RAMS banding profile within palms suggests a single infection event occurs for USR infections. However, in three of the seven BSR infected trees from which isolates had been obtained more than one RAMS profile was evident: Isolates BSR R9 and BSR B1 had distinct banding patterns within an infected tree in plot 88-300, BSR BC B5 was distinct from other isolates within the same tree in plot 86-400 and BSR BD B4 was unique from other isolates within the same palm in plot 86-200 (Table. 2). This indicates that BSR infection can involve more than one isolate and that multiple infections can occur within a single tree.

	420	450	480	500	520	550	580	600	650	690	700	720	750	780	800	850	880	900	910	990	1000	1050	1100	1150	1180	1200	1300	1350	1400	1450
BSR 85-200	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	1
USR 85-200	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1
BSR BB 85-200	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	1
FPA R1 85-200	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	0	0	1	0	0	0	1
FPAR3 85-200	0	0	0	1	1	0	1	0	0	0	1	0	0	1	0		0	0	0	0	0	0	0	0	0	0	1	0	0	1
FPAB3 85-200	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1
FPA B5 85-200	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1
FPA B7 85-200	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	1
USR 86-200	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1
BSR 86-200	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1
BSR BD 1-3 86-200	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1
BSR BD B4 86-200	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
BSR BA 86-400	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1
BSR BB 86-400	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
BSR BC 86-400	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
BSR BD B3 86-400	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1
BSR BC B5 86-400	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1
USR 84-300	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
FPA 84-300	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
FPC2-3B1 84-300	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
FPC R4 84-300	0	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	1	1	0
BSR R9 88-300	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1
BSR B1 88-300	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1
FPA R9 S2 88-300	0	0	0	1	1	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1
FPA R11 S2 88-300	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	1

Table 2. Binary matrix compiled from RAMS profiles of *G. boninense* isolates. Column numbers represent molecular weight of observed bands. Note the same profile from isolates BSR BD B3 and BSR Bc B5 obtained from neighbouring palms in plot 86-400. Different profiles were obtained from isolates within the several BSR infected palms from several plots: BSR tree from 88-300, BSR BC from 86-400 and BSR BD from plot 86-200.

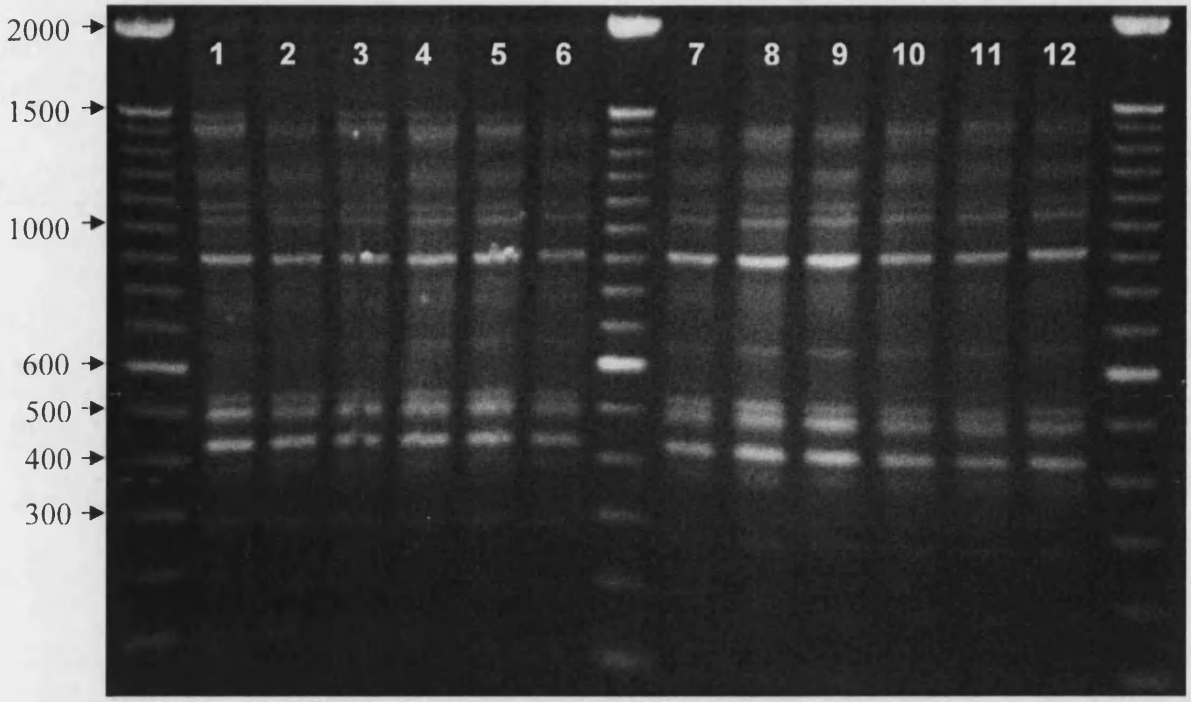

FIG. 32. Microsatellite fingerprints from a single BSR infected tree from plot 85-200. Two lanes represent two PCR reactions for a single *G. boninense* isolate. Lanes 1&2 = BSR R3, 3&4 = BSR R6, 5&6 = BSR R12, 7&8 = BSR B1 S2, 9&10 = BSR B2 S2, 11&12 = BSR B3 S2. Band sizes were estimated using a 100bp ladder (Invitrogen).

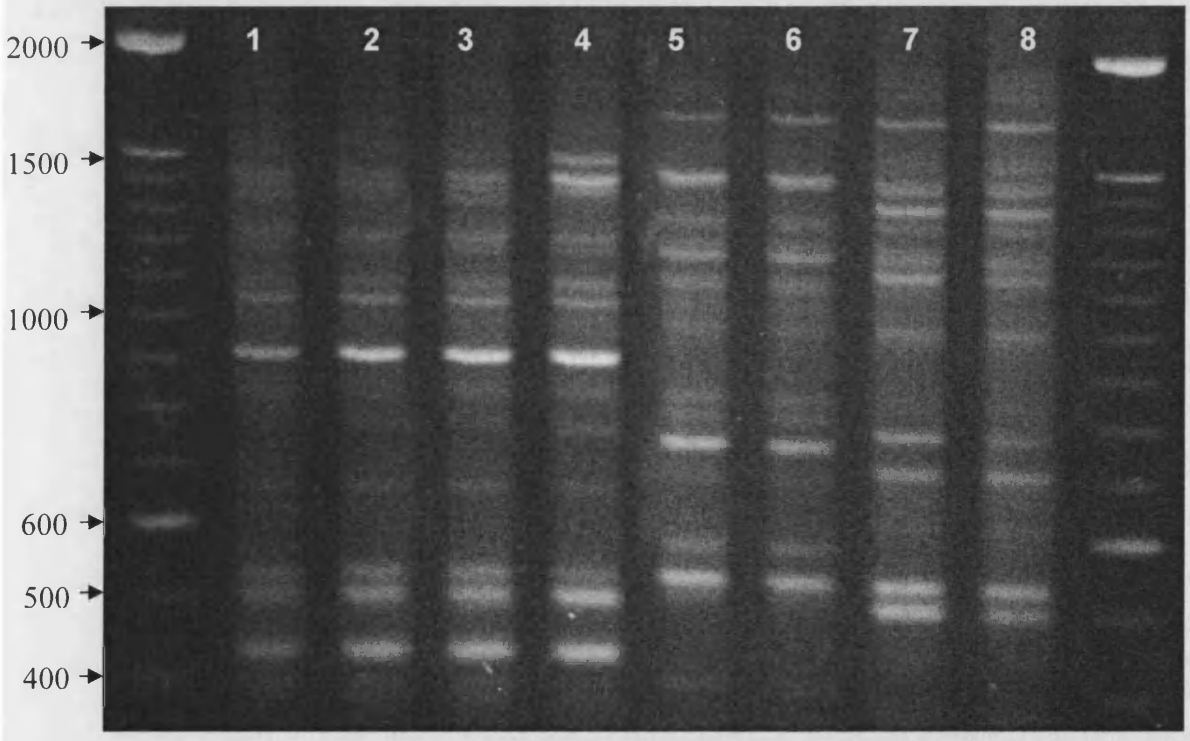

FIG. 33. Microsatellite fingerprints from a single BSR infected tree from plot 85-200. Two lanes represent two PCR reactions for a single *G. boninense* isolate. Lanes 1&2 = BSR B3 S2, 3&4 = BSR B5 S2, 5&6 = FPA R1, 7&8 = FPA R5. Band sizes were estimated using a 100bp ladder (Invitrogen).

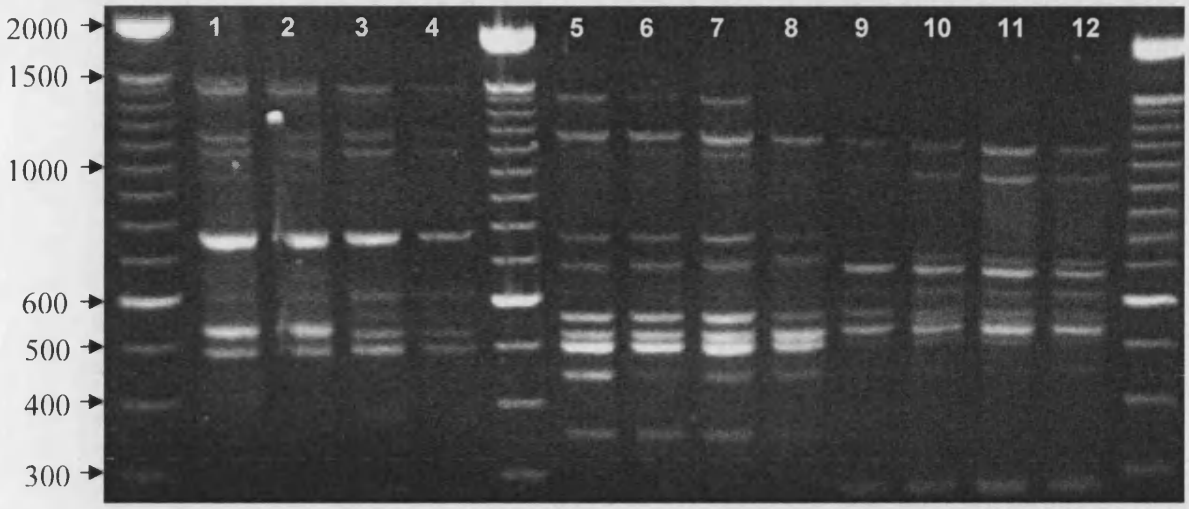

FIG. 34. Microsatellite fingerprints from adjacent BSR infected trees in plot 86-400 and four FPs from plot 84-300. Two lanes represent two PCR reactions for a single *G. boninense* isolate. Lanes 1&2 = BSR BD B3 S2, 3&4 = BSR BC B5 S2, 5&6 = FPA B1 S2, 7&8 = FPA B3 S2, 9&10 = FPC R2 S2, 11&12 = FPC B1 S2. Band sizes were estimated using a 100bp ladder (Invitrogen).

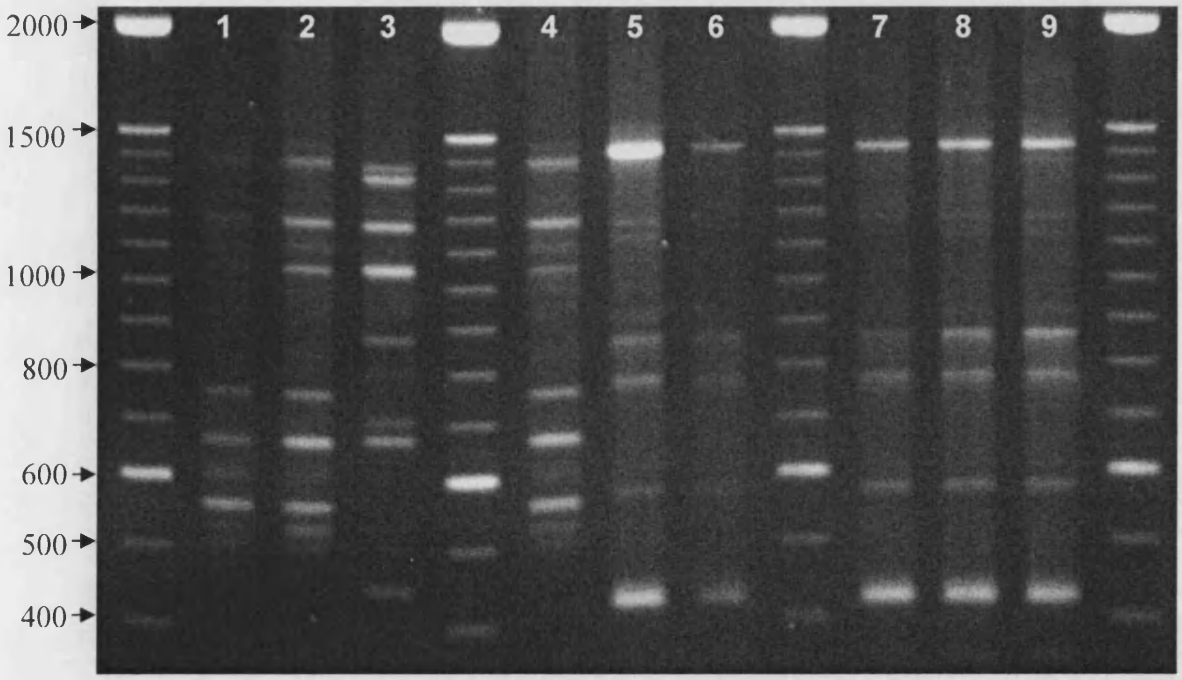

FIG. 35. Fingerprints of *G. boninense isolates* from a USR infected palm and fallen palms in plot 84-300. Each lane is the result of a PCR amplification. Lane 1 = FPC R2 S2, 2 = FPC R3 S2, 3 = FPC R4 S2, 4 = FPC B1 S2, 5 = USR R1, 6 = USR R2, 7 = USR R5, 8 = USR R6, 9 = USR R8. Band sizes were estimated using a 100bp ladder (Invitrogen).

Examination of the matrix using cluster analysis showed that individuals within a single cropping system did not cluster together more than those from different plantings (Fig. 36). In addition, isolates from Sungei Bejanker were equally likely to cluster with isolates from Bah Lias estate as they were to cluster with those within the same estate. For example, *G. boninense* isolates obtained from tree BSR BD in plot 86-200 in Bah Lias, have more similarity based on number of shared bands to isolates obtained from fallen palms, FPA and FPC, from plot 84-300 in Sungei Bejanker. The cluster analysis also shows that the only identical isolates from neighbouring trees are BSR BD B3 S2 and BSR BC B5 S2.

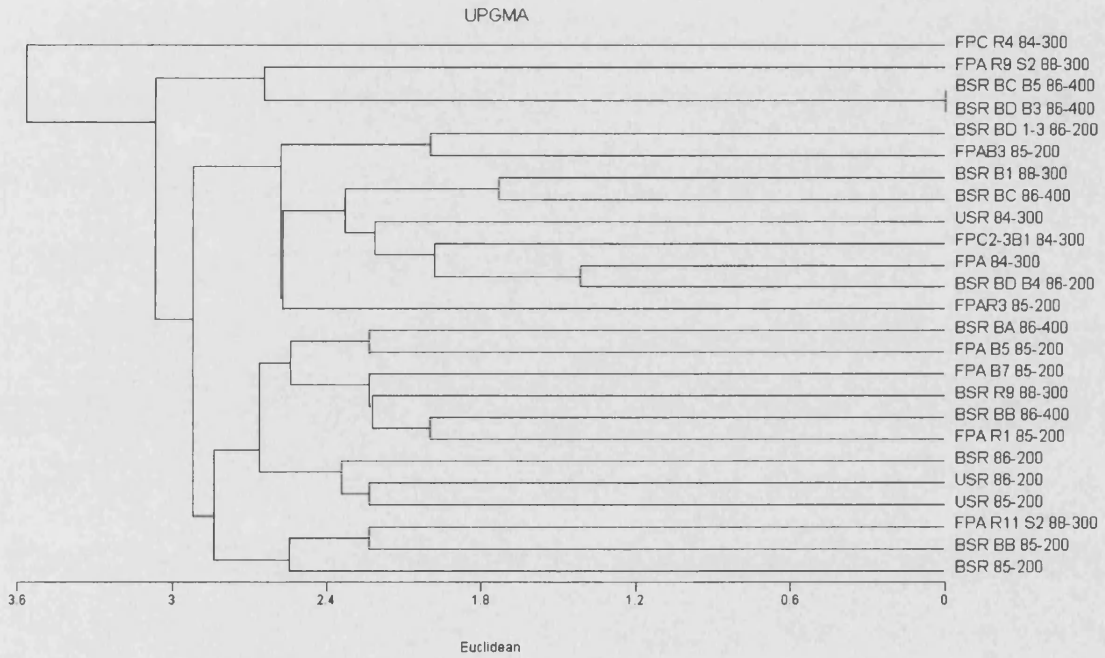

FIG. 36. Dendogram produced by cluster analysis of binary matrix of banding patterns using RAMS.

2.4 Discussion

Field trials in Sumatra showed that large colonised rubber-wood blocks were effective at inducing infection of palm seedlings and that infection rates decreased markedly with inoculum size, suggesting the necessity for a large inoculum source. However, infection studies using three aggressive isolates to infect oil palm under greenhouse conditions showed that a 3 cm^3 inoculum block was sufficient to induce infection in over 80% of seedlings. By immediately establishing intimate contact between oil palm roots and the pathogen, potential for infection was increased. It is probable that in field trials where inoculum blocks are placed directly below roots and subsequently covered in soil, intimate association may not immediately occur and thus chances of obtaining infection are reduced.

This suggests that *G. boninense* may not be able to grow through soil because if mycelium could extend from an inoculum source through the soil, greater rates of infection would be expected. Many *Armillaria* spp. behave preferentially as saprophytes, producing soil invading rhizomorphs that scavenge nutrients (177). However, when rhizomorphs encounter susceptible roots, *Armillaria* root rot may occur. This is a major disease of woody plants and has been identified in numerous regions throughout the world, including North America, Europe, Australia, New Zealand, Kenya and South Africa (42). *Armillaria* species often have a broad host range and two species, *A. mellea* and *A. gallica*, were shown to be have overlapping host ranges and are commonly found on almost all tree species in the mixed hardwood forests of California (20). Rhizomorphs are directly involved in infection of roots of susceptible trees species (150) and Prospero *et al* determined that the most virulent isolates of *A. ostoyae* in Norway were those that produced the greatest number of rhizomorphs (177). In a separate study Morrison determined that all rhizomorph-producing species of *Armillaria* were pathogenic, but those that produced dichotomously branched rhizomorphs, such as *A. ostoyae*, were more virulent than those that produced monopodially branched rhizomorphs (149).

In this study, *G. boninense* appeared to produce monopodially branched rhizomorph-like structures in sterilised soil and FD. However, these structures were not formed in

non-sterilised soil and there are no reports of their occurrence under natural conditions in the field. Thus *G. boninense* does not appear to be soil borne; inability of *G. boninense* to grow through non-sterilised soil suggests that the fungus would be unable to extend from the inoculum source towards the roots in order to infect roots under natural conditions. Therefore, *G. boninense* is unlikely to be able to infect oil palm directly in the same way as *Armillaria* infects its hosts, as it is unlikely to be able to survive saprophytically in soil. This may explain the inability to obtain infection from small inoculum sources using established infection methods (199). It is therefore probable that intimate association between the fungus and oil palm roots is more important for disease establishment than inoculum size.

Reservoirs of *G. boninense* inoculum within oil palm plantings have yet to be fully determined and it remains unclear how long *G. boninense* resting structures can remain viable in the soil or decayed wood. Hasan and Turner (99) tested infectivity of palm trunks by placing bait seedlings at defined distances around trunks and determined that they remained infectious for two years, however it is unknown if *Ganoderma* resting structures in the wood could remain viable for longer than this. Initiation of infection of upper stems remains an enigma and Flood *et al* (77) postulated that debris (FD) collecting behind frond axils may present a rich environment for development of *Ganoderma* inoculum from which invasion of the healthy stem could be initiated. However, as with soil, no *G. boninense* growth was observed in non-sterilised FD in this study, suggesting that *Ganoderma* is also unlikely to be active in FD under natural conditions.

Inability of *Ganoderma* to grow under non-sterile conditions suggests it is a poor competitor and does not support colonisation and proliferation within these environments. This would preclude the possibility of sufficient build-up (free-living) of *G. boninense* inoculum in soil or FD to initiate infections of oil palm. Counter-intuitively, growth of the pathogen was restricted in sterilised soil and FD when roots were added. It is probable that detection of competitive microbes introduced from the rhizosphere, inhibited *G. boninense* growth and possibly induced the formation of resting structures, however anti microbial phytoanticipins possibly present in oil palm roots may also retard fungal growth (161).

Field trials also showed that shading of palms was important for establishment of infection. Markedly reduced infection in exposed palms compared with shaded palms prompted a comparison of rhizosphere temperature between these two treatments. It was shown that the rhizosphere of exposed seedlings regularly reached above 40°C at midday and peaked at 45°C. Cultural studies on pathogenic *G. boninense* isolates showed that hyphal extension of the fungus was severely impaired above 35°C and that prolonged exposure to a temperature of 45°C was terminal. Therefore conditions within a mature stand of oil palm are optimal for *G. boninense* growth and proliferation. Losses are low in young plantings and only become financially significant after about 15 yrs (180). Canopy closure creates shaded conditions, maintains humidity and minimizes temperature extremes, which may account for the low initial disease incidence and the eventual high levels observed in mature stands.

The development of USR infections is still unclear, but is possibly brought about by opportunistic establishment by basidiospore(s) on wound surfaces and eventual progression into the main stem, causing infection. The fungus responsible for root rot of conifers and pine, *H. annosum* may also potentially cause direct infection of stems. Some conifers show symptoms of wound infection and *H. annosum* has been recovered from these; although an extremely rare phenomenon, Redfern and Stenlid suggests limited infection by this route cannot be discounted (182). Nevertheless, the most common source of infection by *H. annosum* is from basidiospore (or conidiaspore) colonisation of cut stumps and subsequent invasion into the senescing root system, from where it progressively invades the root system of living trees (17).

In this study, investigation of infection on roots of oil palm seedlings showed that dikaryons were required for infection, which concurs with earlier experiments conducted at Bah Lias research station (BLRS) (Yonnes Hasan, pers. comm). This would mean that for basidiospores to cause direct infection, anastomosis of two compatible germinating spores would be essential. Spores from other fungal tree pathogens are known to infect trees through wound sites. The ascomycete, *Cryphonectria parasitica* is an aggressive pathogen of chestnut trees including (European sweet chestnut) and *Castanea dentata* (American chestnut). Airborne spores colonise wound sites on the bark and grow, into the inner bark and cambium and progressively spread, around the trunk and girdles the trunk, killing all parts of

the tree above that point (156). However, Rayner and Boddy (181) consider that infection of woody tissue by minute inoculum represented by basidiospores can only be expected to occur under severely circumscribed conditions.

The possibility of infection of oil palms by *G. boninense* mediated by insect vectors has not been studied, but insects have been shown to be involved in the colonisation of wound sites on spruce trees in Northern Europe by *Amylostereum areolatum*. *A. areolatum* is a saprophytic fungus usually occurring on fallen trunks and stumps of *Picea abies*, but is also regarded as an important source of wound decay, usually infecting 5-20% of open bark wounds on living trees (241). The *Sirex* woodwasp is thought to carry *A. areolatum* in the form of mycelium fragmented into oidia or arthrospores to wound sites, where they colonise and eventually develop into an active rot within the damaged stems. The possibility that an insect vector, such as *Oryctes rhinoceros*, may carry dikaryotic *G. boninense* in the form of fragmented mycelium into wound sites on oil palm trunks is a possibility that has not as yet been investigated.

Currently basidiospores are believed to be the major factor influencing increased incidence of USR, but there have been no reports of successful infection of oil palm using a basidiospore inoculum and optimal conditions for their germination in the field has not been determined. At BLRS, Hasan *et al* (98) attempted to directly infect oil palm trees with highly concentrated spore suspensions. These were either painted onto cut fronds or injected directly into the trunk, however, both experiments failed to induce infection and there are currently no reports of successful infection of oil palm by basidiospores. However, this study has shown for the first time that *G. boninense* basidiospores can germinate readily on wounded palm surfaces. Extensive contamination of wound sites by other fungi and bacteria was also detected, and these may inhibit colonisation by *G. boninense*, particularly of the nutrient-rich peduncles. It is possible that basidiospores could be pulled within xylem vessels by negative pressure on wounding, which would reduce the amount of competition basidiospores are exposed to during germination. Basidiospores that are pulled into xylem vessels would also be exposed to less solar radiation, which has previously been shown to inhibit germination of fungal spores (186), and therefore have a better chance of germinating and causing infection. Panchal and Bridge (162) managed to amplify

Ganoderma DNA from 0.25-1 cm below the surface of cut fronds using *Ganoderma* specific primers (GanET), though it is uncertain if amplification was from potentially inert basidiospores or vegetative growth. It was noted that positive results did not always persist and of the ten palms that had positive amplification of *G. boninense* DNA, only six tested positive after two years and only four ever showed symptoms of disease. Estimation of vessel lengths in this study suggests that basidiospores could potentially be pulled as much as 10 cm from the cut surface, and therefore positive DNA amplification less than 1 cm from the surface could easily be from basidiospores, which may or may not be viable.

Basidiospores did not germinate on cut fronds in the first experiment and the reason for this is unclear. *In vitro* germination proved that most of the basidiospores were viable and so a proportion of the basidiospores would have been expected to germinate. However, analysis of *ca.* ten samples comprising thousands of basidiospores, revealed no germination. This could result from a number of factors; numerous bacterial and fungal competitors were seen in many of the samples and could be inhibitory; immediate application to wound sites may have meant exposure of spores to phytoalexins and other host defence responses stimulated by frond wounding; abiotic factors such as climate, sunlight and humidity may prove important for viability of spores under natural conditions. Elucidating the conditions under which *G. boninense* basidiospores can germinate may be important for management of disease; if basidiospore germination is influenced by abiotic factors, local application of fungicide or biocontrol agen to cut fronds and peduncles at certain times of the year may be useful as a control strategy for USR.

It is possible that the length of time that basidiospores are kept in suspension may also affect viability and infectivity under natural conditions. Basidiospores, and fungal spores in general, are produced in a water-soluble mucilaginous matrix containing numerous proteins and glycoproteins. This matrix is believed to play a role in survival, differentiation, growth and pathogenicity of fungi and this matrix can be removed when diluting spores in water or buffer for infection studies (133). To date, no successful direct infections oil palm resulting from inoculation of basidiospores have been reported. Mahaku and Goodwin (133) suggest that preparation of fungal spores for infection studies could be conducted in buffers containing mucin. Mucin is

a heterologous group of highly glycosylated glycoproteins that are chemically similar to fungal spore glycoproteins and preparation of spore suspensions using mucin showed significantly increased germination in spore suspensions from 7/10 phytopathogenic fungi tested, including the basidiomycete *H. annosum*.

An estimate of spore production was made by Sanderson (193) where a glass slide with sticky tape was placed directly below the pore surface of an active bracket over a period of time. This provided an estimate of over 2 million spores produced by a 100 x 50 mm bracket in daylight hours. However, airborne basidiospore levels had not been assessed within plantations and therefore it was unknown where and when the greatest basidiospore concentrations occurred. Therefore, atmospheric basidiospore levels from different locations at Bah Lias estate, North Sumatra, were observed using a centrifugal air sampler and counts were made by light microscopy. It was impossible to differentiate spores produced from different *Ganoderma* species microscopically, as these often have very subtle differences (175). However, most of the *Ganoderma* basidiophores within plantations are from the species *G. boninense* and the large vacuole present in *Ganoderma* basidiospores was used to discriminate them from contaminating spores from other fungi.

Spore counts were lowest in the early morning rising to a peak in the early evening, before again reducing overnight. It therefore appears that light intensity may play a role in release of basidiospores. *Ganoderma* basidiospores were in greatest abundance in the mature stand and were lowest in the three year old windrow. High basidiospore production over a period of years from mature stands may therefore influence the incidence of USR in neighbouring juvenile stands. Sampling of a mature windrow revealed low aerial basidiospore levels, suggesting that windrows may not be as important for basidiospore production as mature plantings. However, windrows are more exposed and wind dispersal may be responsible for wider dispersal of the fungus. Sampling using the biotester has provided an insight into levels of spore production within three different locations but time constraints restricted sampling in this study. Further sampling could reveal the effect of spore production from mature plantings on spore numbers within juvenile plantings, by comparing plots of juveniles bordering on rubber or plantings adjacent to mature plantings. Also further testing should be done to determine the extent of spore

production within one year old windrows over a period of days, in case of variation due to air currents.

Analysis of epidemiology using sequencing and fingerprinting of *G. boninense* isolates sampled from fallen palms, BSR infected standing palms and USR infected standing palms was also conducted in this study. Sequencing of highly variable intergenic spacer (ITS) sequences has been used for numerous *Ganoderma* taxonomic studies (87, 146, 209) and provided the sequences for identification of species. Sequencing of ITS regions from rDNA was sufficient for species recognition and all isolates obtained from infected standing palms in Sumatra were shown to be *G. boninense* after BLAST analysis. This supports the view of Pilotti *et al* who determined that only *G. boninense* causes disease of oil palm, based on morphological characters (175). Sequencing also demonstrated that some of the *Ganoderma* sampled from FPs in Sumatra were species other *Ganoderma* spp., presumably living saprophytically on oil palm material.

Basidiospores are present in large numbers within the atmosphere of mature stands and are implicated in direct infection of upper stems. Yet, USR incidence is relatively low in comparison to BSR, suggesting that infections of upper stems are likely to be chance events where conditions favour the pathogen. Analysis of *G. boninense* isolates using RAMS revealed that individual USR infection only contained a single isolate of *G. boninense*, which might be expected if infection events in upper stems are exceptional events.

Conversely, analysis of isolates present within BSR infected palms showed that 3/7 trees tested contained more than one isolate of *G. boninense* based on presence of different RAMS profiles. BSR infections may therefore contain genetically distinct individuals within an infected tree. This concurs with a previous study by Miller *et al* who used mtDNA RFLP using 4-base cutter restriction endonuclease *Msp*I in Malaysia (143). They found that two BSR infected palms, 103/SB and 219/SB, contained isolates with different RFLP profiles, suggesting infection by more than one isolate.

If basidiospores were critical infectious agents for both BSR and USR infections then a similar a pattern of decay would be expected for both. This was not the case and examination of bole tissue from asymptomatically infected palms showed multiple infection lesions after scraping off the roots. This suggests that many roots had contacted an inoculum source and been infected at approximately the same time. Since roots explore soil in all directions it is possible that they will contact more than one source of inoculum, which could result in simultaneous infection by more than one isolate of *G. boninense* and thus contain multiple infections in a single palm.

Molecular evidence for mycelial spread of the disease from FPs or BSR infected palms to neighbouring palms was not strong in this study. There was no evidence to link BSR infected trees to neighbouring FPs, as no identical RAMS profiles were observed in both FPs and adjacent BSR palms. Similarly, none of the banding patterns from FPs was shared with isolates obtained from adjacent FPs, indicating a genetically variable population and the importance of basidiospores, which concurs with previous studies in Malaysia (142) and Papua New Guinea (171, 173). However, analysis of isolates from within two adjacent BSR infected palms (plot 86:400 from Sungei Bejanker) did show identical profiles, indicating vegetative spread of the pathogen. Miller *et al* recorded similar findings (142) where adjacent BSR palms, 102SB and 103SB, contained the same mtDNA RFLP band pattern. These two studies show that genetically identical individuals were present in adjacent palm trees, and this is extremely unlikely to occur if infection of the palms had been by basidiospores as the mating system in *Ganoderma* favours outcrossing (4, 174) and even if two compatible spores from the same bracket did infect a neighbouring palm, meiotic recombination would likely distort the genetic fingerprints.

Although some of the findings point towards mycelial infection, Miller *et al* (142) and Pilloti *et al* (171, 173) argue that the general high genetic variability within plantations is evidence against vegetative spread of the pathogen. However, high genetic variability of *G. boninense* populations within plantations undoubtedly leads to variation within toppled palms in the field. Numerous genetically distinct individuals may infest trunks left in the field and roots that contact the inoculum source may become infected by such genetically distinct individuals. This has been demonstrated by studies conducted on the pathogen responsible for pine root rot (*H.*

annosum) and the biocontrol fungus *P. gigantea*. Highly efficient spore dispersal by *P. gigantea* results in a single pine stump being colonised by several genetically distinct individuals, based on morphological characters, pairing experiments and RAMS fingerprinting (238). Similarly, Swedjemark and Stenlid (219) showed that 27 genets of *H. annosum* were isolates from within a single pine stump over a two-year period based on somatic incompatibility studies. Therefore, it seems reasonable to assume that based on the extremely large quantity of spores within the atmosphere of palm plantations and the size of an intact, toppled oil palm trunk, that a single trunk may become colonised with a large number of genetically distinct isolates.

The importance of basidiospores in dissemination of disease was shown when cluster analysis was conducted on the binary matrix produced from banding patterns. This showed that isolates from a single plot often did not cluster together and that isolates from a plot in Bah Lias estate confirmed previous experiments where a high genetic variability was observed within oil palm cropping systems in Malaysia (143) and PNG (176). The diverse nature of *G. boninense* populations within oil palm cropping systems highlights the importance of basidiospores in *G. boninense* populations. Flood *et al* proposed a lifecycle that introduced the significance of basidiospores in saprophytic and pathogenic cycles (77). It seems clear that basidiospores play important roles in disease epidemiology and genetic variation within plantations implicates their involvement in USR infections. Sanderson *et al* postulate that involvement of basidiospores in the infection process will result in segregation of aggressive characters and that selection pressures are responsible for the earlier detection of BSR and USR in Indonesia and Malaysia (195).

Nonetheless, their role in BSR development remains unclear and compelling evidence for root infection has been shown in this study and previous studies (99). Investigation into epidemiology of *Ganoderma* stem rots of oil palm appears to have reached a paradox: high genetic variability between infections of neighbouring palms suggests direct infection by basidiospores, whereas controlled infection of seedlings and field observations of BSR infected palms suggest root infection. Root infection of pine trees in temperate regions by *H. annosum* typically results in a single genet extending over a large area (255), lending weight to the theory of direct infection of palms by basidiospores. However, the two principles are not mutually exclusive.

High genetic variability of *G. boninense* between adjacent trees is undoubtedly a consequence of spore dispersal and basidiospores have been shown to be present in very large quantities in the atmosphere of plantations and probably germinate in great numbers on wood debris left in the field, particularly large oil palm trunks. These may then serve as infection foci within plantations for infection of roots by vegetatively growing hyphae.

Basidiospores appear to be the driving force for dissemination of *Ganoderma* and ultimately increasing disease incidence. In Papua New Guinea, a zero tolerance policy is aimed at removal of all basidiophores within plantations in Milne Bay estates (107, 194). *Ganoderma* stem rot has yet to become a major problem in PNG, making this task more achievable. However inoculum reduction is also desirable in Malaysia and Indonesia where USR incidence has become problematic, particularly in seed gardens in North Sumatra (98). One way to facilitate this could be for harvesters to disrupt basidiophores as they work in the field and a reduction in basidiospores could reduce USR within plantations and may have an effect downstream on incidence of BSR.

3 *Ganoderma* Pathogenicity and Wood Decay

3.1 Introduction

3.1.1 Wood Decay

There are three distinct types of wood decay: i) brown-rot, ii) soft-rot and iii) white-rot; the latter is the most common and biologically important (181). Brown-rot fungi represent a small minority of basidiomycete fungi, preferentially attacking the cellulose component of wood with no extensive lignin degradation. Transmission electron microscopy of tissue decayed by brown-rot fungi often shows intact cell wall structure and darkly staining middle lamella with intact lignin component. However the cell wall is often wavy with areas of secondary wall completely removed following degradation of cellulose. This weakens the structure and when pressure is applied to blocks decayed by brown-rot fungi *in vitro* the wood appears brittle. These fungi can cause severe damage in buildings, compromising the structural integrity of wooden support beams and is termed 'dry rot' (181). They do not possess oxidative phenoloxidase or peroxidase enzymes and *in vitro* decay studies show brown-rot fungi induce less dry weight loss of wood than white-rot fungi (2).

Soft-rot fungi are the least studied wood decay fungi due to their limited potential for biotechnology. Species that cause soft rot belong to Ascomycetes (or Deuteromycetes), including *Penicillium*, *Thielavia* and *Trichoderma* species. They attack the polysaccharide component of wood in water-saturated conditions such as bogs and swamps but are of little consequence elsewhere due to competition from brown and white-rot fungi.

White-rot fungi degrade all components of wood and many species have been observed to degrade lignin selectively (9). Production of lignin-degrading enzymes and enzymes for other recalcitrant compounds has led to interest in these fungi for biotechnological purposes. For example, they may be used for wood chip treatment in biopulping in the paper industry to improve paper quality and reduce energy costs.

White-rot fungi also have the ability to degrade recalcitrant pollutants such as PCB, chlorinated phenols and other industrially generated compounds (6). For biotechnology purposes fungi must meet certain requirements such as, ability to survive at elevated temperatures, asexual reproduction and a fast growth rate; *P. chrysosporium* satisfies these criteria and is currently the most studied white-rot basidiomycete (37).

Brown-rot fungi most often occur on wood of gymnosperms, whereas most wood decay fungi on angiosperms are white-rot basidiomycetes (2). Angiosperm wood has a lower lignin and higher holocellulose content than gymnosperms but there are also differences in the lignin polymers. Gymnosperm lignin contains predominantly guaiacyl subunits (G units) synthesised from the precursor coniferyl alcohol, whereas angiosperm lignin contains both syringyl subunits (S units) synthesised from the precursor sinapyl alcohol and G units in similar quantities (250). Decay studies using the white-rot fungus *Phanerochaete chrysosporium* indicated reduced ability of the fungus to degrade the guaiacyl lignin of gymnosperms compared to the guaiacyl-syringyl lignin of angiosperms (2). Several species of *Ganoderma* were used to degrade high-density hardwoods including silver leaf oak and white fir (a conifer) and showed on average 35% weight loss of oak (angiosperm) compared with 14.4% weight loss of white fir (gymnosperm). The greater weight loss from angiosperm wood was attributed to the more facile breakdown of guaiacyl-syringyl than guaiacyl lignin by white-rot fungi (7).

3.1.2 *Ganoderma* Wood Decay

Ganoderma species are white-rot basidiomycetes with worldwide distribution. The genus contains both pathogenic and non-pathogenic species but most are saprophytic, living on dead wood material in natural forests. *Ganoderma* species have varying host ranges, *Ganoderma lucidum* occurs on many hardwoods throughout the United States, *G. tsugae* is primarily found on *Tsuga* spp. but also occurs on *Abies*, *Pseudotsuga*, and occasionally *Betula* spp. In contrast, *G. colossum* occurs on relatively few hardwoods, one Central American conifer and palms in the Gulf Coast region of the United States. *G. zonatum* has a more restricted host range, occurring only on palms of a number of genera (7).

All *Ganoderma* spp. are white-rot fungi, but some species have been observed to delignify wood selectively, whilst others are simultaneous wood degraders. For example, a phenomenon in temperate rainforests of Southern Chile termed 'Palo podrido' involves extensive lignin degradation resulting in wood that is 70% digestible and is used by local people as animal feed (9). Over 70% of wood samples from the region yielded *Ganoderma* species and *G. australe* was identified as the most important (135). Another temperate species of *Ganoderma*, *G. tsugae*, was found to induce selective delignification of eastern hemlock in the United States (28). However, many *Ganoderma* spp. are simultaneous wood degraders, concurrently removing similar proportions of lignin, cellulose and hemicellulose. *In vitro* studies have shown that wood type may affect the nature of wood degradation by these fungi (6). Additionally, some species appear to show both selective lignin degradation and simultaneous decay depending on the type of wood (9). For example, analysis of wood decay in field samples, showed that wood with low lignin content and predominance of guaiacyl-syringyl residues favoured selective delignification (9). However, the 'Palo podrido' phenomenon was not replicated under laboratory conditions suggesting that other factors contribute to the type of wood degradation observed in natural environments (135). Low nitrogen levels in natural forests compared to commercial forests are postulated to be a factor in greater lignin peroxidase production and thus prevalance of selective delignification in these ecosystems (56). Moisture levels, oxygen levels and polysaccharide composition may also influence the wood degrading process (27).

3.1.3 Lignin: Structure and Degradation

Phenolic lignin polymers are particularly abundant in wood cell walls and contribute to compressive strength, resistance to degradation by microbial attack and water impermeability to the polysaccharide-protein matrix of the cell wall. Biosynthesis of lignin progresses via the free radical forms of precursors: *para*-coumaryl, *p*-coniferyl and *p*-sinapyl alcohol (250). In the final polymer they form *p*-hydroxyphenyl, *p*-guaiacyl, and *p*-syringyl type units attached predominantly by arylglycerol-β-aryl ether linkages (Fig. 37&38). Several forms of lignin are known and the precise regulation and monomer composition during development and stress responses

strongly implies that alternative lignins have different adaptive functional properties (35). Under laboratory conditions, none of the white rot species studied were able to grow with polymeric lignin as the only carbon source, suggesting that lignin breakdown primarily serves to gain access to plant carbohydrates (168).

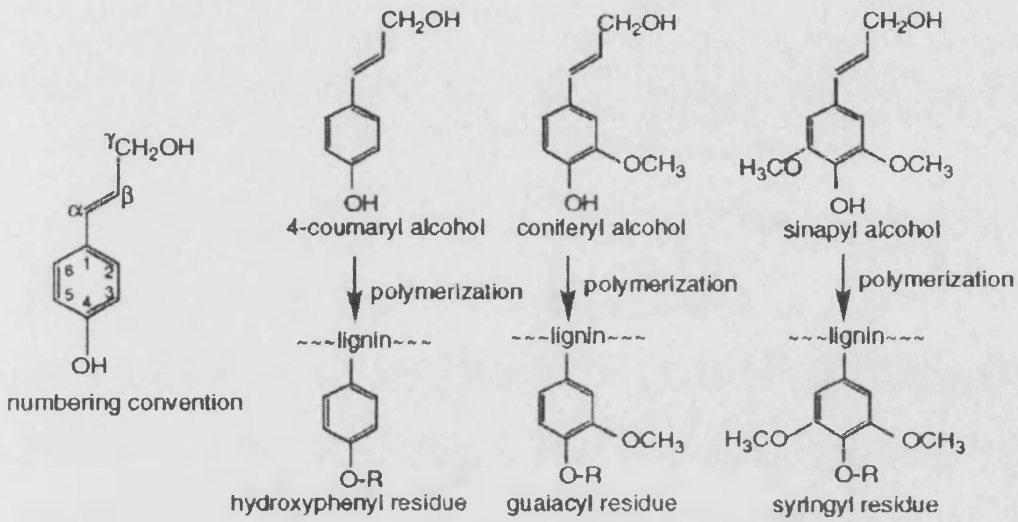

FIG. 37. Structure of the three lignin precursors and the residues derived from them. Numbering convention is shown on the left (250).

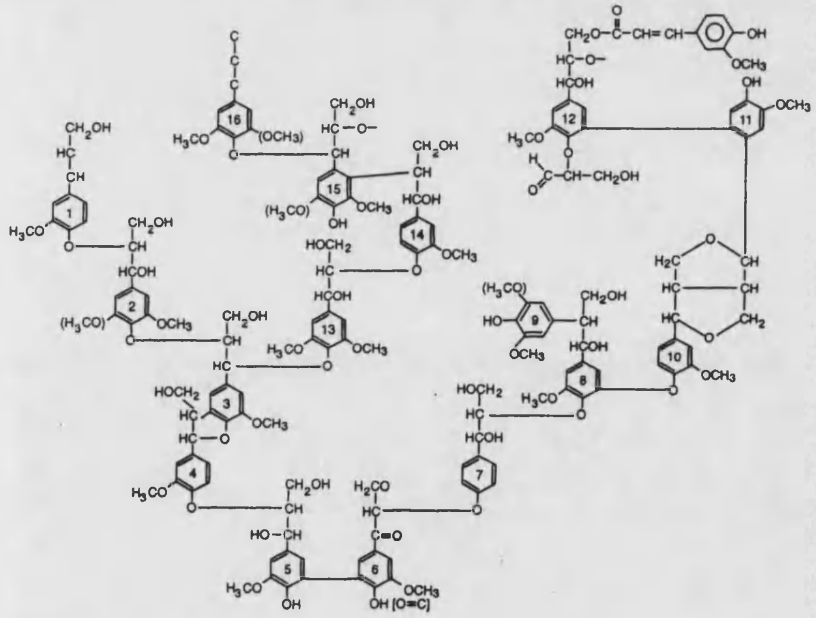

FIG. 38. Schematic structure of lignin. Phenylpropene subunits are linked together with ether and carbon-carbon bonds (168).

Due to the irregular nature of the lignin polymer, degradative enzymes are oxidative, and non-specific. The three major families of fungal lignin modifying enzymes are lignin peroxidases (LiP), manganese-dependent peroxidases (MnP) with H_2O_2 as electron acceptor, and laccases with O_2 as the electron acceptor (151). Lignin peroxidase, MnP and laccase have been the focus of recent study due to their ability to degrade potential pollutants and to carry out environmentally clean processes such as biopulping and bleaching of pulps (58). Laccases, LiPs and MnPs can oxidise phenolic compounds, thereby creating phenoxy radicals, while nonphenolic compounds are oxidised via cation radicals. Laccases oxidise aromatic compounds with relatively low ionisation potentials, whereas compounds with higher ionisation potentials are readily oxidised by LiPs (57). Investigations by Kersten (112) and Hammel *et al* (90) established the basis for ligninase oxidation: susceptible aromatic nuclei are oxidised by one electron producing unstable aryl cation radical species, which undergo a variety of non-enzymic reactions. These radicals can either form new linkages with other lignin subunits, which results in polymerisation or turn into quinones or ring cleavage products. These compounds can probably be catabolised further or taken up by the fungus, which shifts the reaction balance towards depolymerisation (168).

Electron microscopy suggests the initial agent of delignification must be able to diffuse into the wood cell walls. MnP and LiP are large, heme containing enzymes and the molecular weight of these suggests that they do not readily penetrate into sound wood, since the pores of wood would not be large enough for penetration by the enzymes (27). This has led to suggestions that initial delignification agents are low molecular weight compounds. Since LiP contains protoporphyrin IX, some researchers have used synthetic metalloporphyrins to degrade lignin model compounds as well as lignin in wood *in vitro* and low molecular weight porphyrins have been shown to cause delignification in a manner similar to white-rot fungi (179). However, hemicellulases may also aid lignin degradation by degrading hemicelluloses in the secondary cell wall, thus creating channels of sufficient size to facilitate access to LiP and MnP (27).

Regulation of lignin-degrading enzymes is still not fully understood but the majority of white rot fungi investigated, including *P. chrysosporium*, are shown to initiate

lignin degradation when nitrogen, carbon or sulphur are limiting (86). *In vitro*, addition of the detergent Tween 80 (125) and tryptophan (44) to liquid cultures results in enhanced activity of LiP in *P. chrysosporium*. Whereas Tween 80 is thought to stimulate production of LiP, reverse transcriptase-PCR showed that tryptophan does not induce LiP at the expression level, but rather is likely to serve a protective function against H_2O_2 inactivation (44). Veratryl alcohol is not thought to perform a protective function as the normal rapid decrease in LiP activity was observed in ageing cultures; however, increased activities in cultures supplemented with veratryl alcohol implicate this with having an induction type of mechanism (73, 74). Similarly, d'Souza *et al* (58) used commercially available syringic acid as a substitute for syringyl residues present in many wood lignin polymers as a supplement in liquid cultures of *G. lucidum* and found that this induced laccase production with activity comparable with those from liquid cultures supplemented with cell walls.

3.1.4 Cellulose: Structure and Degradation

Cellulose is the major structural component of plant cell walls and is visible by electron microscopy as microfibrils of *ca.* 10 nm diameter (45). It is a linear molecule constructed from long chains of β-1,4 linked glucose units (FIG. 39). The degree of polymerisation is variable but is estimated to be in the region of $2\text{-}14\text{x}10^3$ glucose units, giving the molecule a molecular weight of at least $1\text{x}10^6$ Da and a length of 1-7 μm (89). Cellulose is distinguishable from other plant cell wall components by its high degree of crystallinity and its relatively homogeneous chemical composition. Primary cell wall molecules tend to be shorter and more disperse than those in the secondary cell wall. The rigid and linear nature of the molecule and the unsubstituted nature of the carbon atoms means that the molecules can become closely aligned parallel to their long axis and hydrogen bonds can form between molecules, resulting in microfibrils (140). The crystalline nature of the molecule excludes water and makes it relatively resistant to enzymatic degradation and conveys much of the strength to the cell wall.

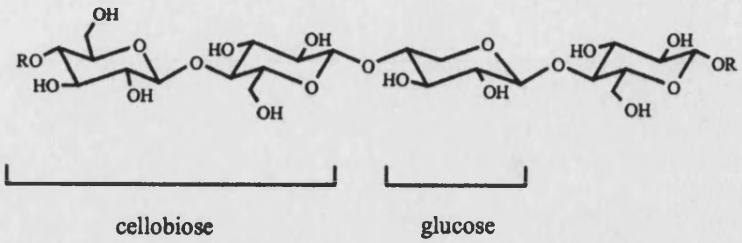

FIG. 39. **A schematic structure of the cellulose chain (140).**

Cellulose degradation is brought about by the action of a complex of cellulase enzymes shown in Fig. 40. Cellulases are glycoproteins containing a carbohydrate moiety that has a role in binding the substrate (61). Three major enzymes are involved in the breakdown of cellulose to glucose: endoglucanase (endo-1,4- β-D-glucanase, EG, EC 3.2.1.4), cellobiohydrolase (exo-1,4- β-D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1,4- β-D-glucosidase, BG, EC 3.2.1.21) (190). Endoglucanases (EG) attack by hydrolysing β-linked bonds within non-crystalline regions (amorphous) and regions of less ordered crystalline cellulose, thus opening up cellulose chains. CBH binds edges of crystalline cellulose towards the reducing end, cleaving cellobiose units from the non-reducing end. β-glucosidase converts cellobiose and short chain cellodextrins to glucose (61). Multiple versions of EG and CBH have been identified; genes encoding several isoforms of EG and CBH were identified in *Trichoderma reesei* (225) and multiple forms of CBH have been studied in *Talaromyces emersonii* (231) and *Mucor circinelloides* (190).

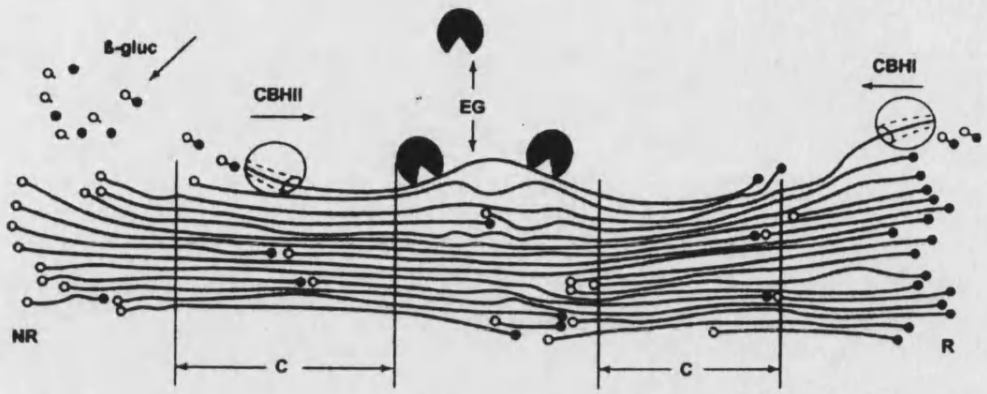

FIG. 40. A schematic view of cellulose and action of CBHs, EGs and β-glucosidase. C defines the highly ordered crystalline cellulose, R the reducing ends, and NR the non-reducing ends. EG predominantly attacks the amorphous cellulose structures and β-glucosidase produces glucose from cellobiose (224).

In general, fungal cellulase systems are induced by presence of cellulose or its breakdown products and are repressed by by the presence of low molecular weight carbon sources that are more readily metabolised than cellulose (catabolite repression) (140). Cellulase regulation in wood decay fungi can vary greatly depending on the type of decay. In many white-rot fungi these enzymes are induced by the presence of cellulose or cellobiose. In brown rot fungi, cellulases are constitutive and are produced even in the presence of the end product. In other types of decay, cellulolytic enzymes can be controlled by end product or intermediate product repression and by the state of equilibrium (61).

3.1.5 Hemicellulose: Structure and Degradation

Hemicellulose comprises complex molecules consisting of a backbone of 1,4-β–D-xylans, 1,4-β-D-galactans or 1,4-β–mannans. In wood hemicellulose molecules are inderdispersed with amorphous regions of cellulose and surround crystalline cellulose. Covalent hemicellulose-lignin bonds involving ester or ether linkages form lignin-carbohydrate-complexes in the S2 cell layer and degradation of these polysaccharides is postulated to be required for initiation of lignin degradation (247). Xylan polysaccharides are the major hemicellulose components in many plant cell walls. The backbone may carry various substituents, the nature of which depends on the plant cell from which the polysaccharide originated. Typical substituents are 1,3

linked α-L-arabinose and α-1,2-linked 4-O-methyl glucuronic acid residues, which are attached glycosidically to the backbone, and coumaric, ferulic and acetic acids which are attached by ester links (254). These side groups render hemicelluloses noncrystalline, so that they exist more as a gel than as oriented fibres.

Enzymatic degradation of hemicelluloses requires a complex set of enzymes reflecting the variability of the hemicellulose structure (Fig. 41). Hydrolysis of the polysaccharide requires 1,4-β-D-xylanases (E.C. 3.2.1.8), 1,4-β-D-mannanases (E.C. 3.2.1.78), 1,4-β-D-galactanases (E.C. 3.2.1.90) that hydrolyse the glycosidic linkages in the backbone of the polymer (61). Endo-β-1,4-xylanases catalyse the random hydrolysis of β-1,4-glycosidic bonds in xylans, whereas endo-1,4-β-D-mannanases hydrolyse 1,4-β-mannopyranosyl linkages of gluco and galactoglucomannans. Side chains are cleaved by a number of different enzymes including α-L-arabinofuranosidases, acetyl xylan esterases, ferulic and *p*-coumaric acid esterases (254). Regulation of hemicellulase genes appears to be less strictly regulated than cellulase, with reports of both constitutive and inductive enzyme production (70).

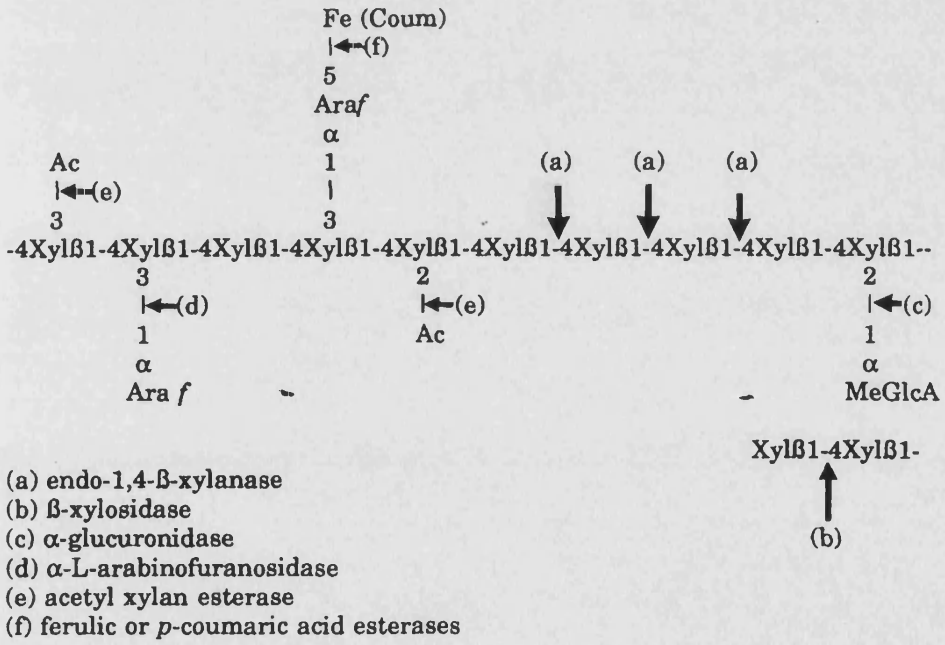

FIG. 41. Xylan hemicellulose and sites of attack by xylan degrading enzymes. Ac, acetyl group; Ara*f*, L-arabinofuranose; MeGlc, 4-O-methyl-D-glucuronic acid; Xyl, D-xylose.

3.1.6 Other Plant Cell Wall Components and Starch

Pectin is a common name for a group of amorphous polymers that exist predominantly in the middle lamella of the plant cell walls. They are rich in α-1,4-galacturonic acid, which can be methyl esterified or in a free acidic form. Polygalacturonic acid polymers also contain neutral sugars, D-galactose, L-arabinose, L-rhamnose, D-xylose, L-fucose and D-apiose. Calcium forms crosslinks in pectin that affects the structural properties of the amorphous matrix. In woody tissue pectin is often replaced in the middle lamella by lignin and contributes to less than 1% of the cell wall components in lignified soft and hardwoods (61). Therefore pectin is likely to play a minor role in oil palm infection by *Ganoderma*, but may be a readily utilised carbon source for initial degradation of palm wood. It may also be an important carbon source during infection of juvenile palms, as pectin is likely to be found in greater proportion in the middle lamella of seedlings. Three major enzymes are involved in its degradation and are shown in Fig. 42.

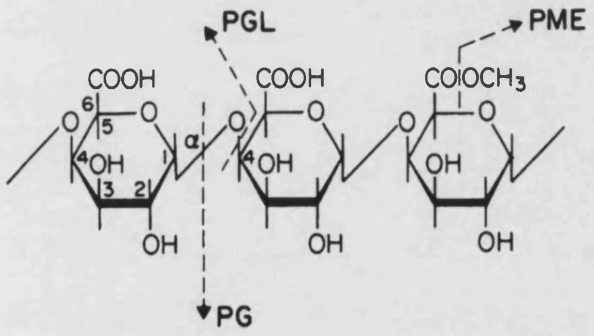

FIG. 42. Polygalacturonide and its enzymatic cleavage. Endo-polygalacturonase (PG) and endo-polygalacturonide lyase (PGL) cleave the α-1,4-galacturonide chain and demethoxylation is by pectin methylesterase (PME) (46).

Starch is the most abundant storage polysaccharide in plants and is usually deposited as large storage granules in the cytoplasm of cells. It consists of α-1,4-bonds with some α-1,6-linkages forming branches to the main chain. Numerous enzymes are involved in starch degradation, including α-amylase, pullulanase and glucoamylase amongst others (168) (Fig. 43). Starch is present in large quantities in mature date palm tissue (2); if similar high quantities are present in oil palm, this would represent a major and easily utilisable food source to provide energy for degradation of more recalcitrant lignin.

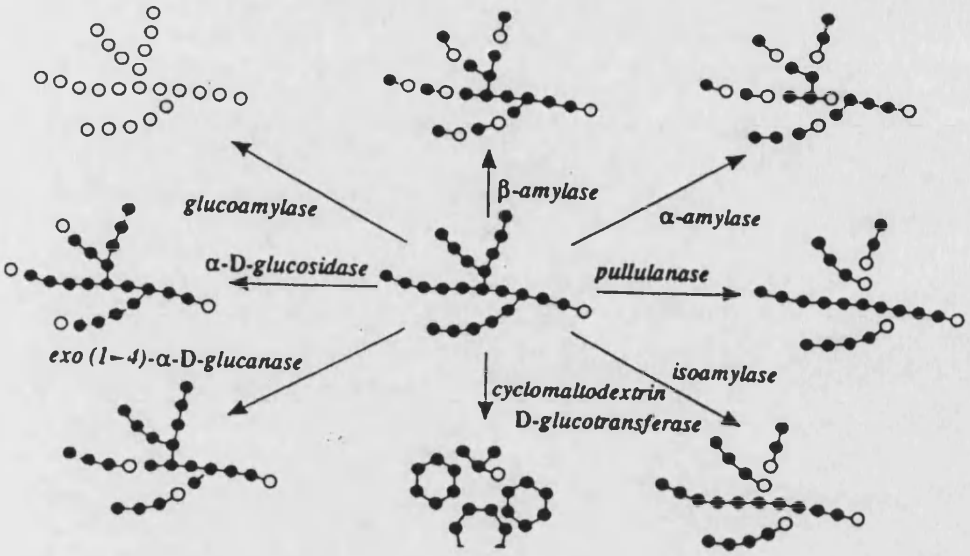

FIG. 43. **Schematic representation of α-1,4-linked and α-1,6-linked glucose and mode of action of starch degrading enzymes (168).**

3.1.7 Invasion of Living Tissue in Trees and Woody Species

Despite the commercial importance of many tree species knowledge of host-pathogen interactions lags behind that of herbaceous plants where understanding is becoming more advanced at the genetic level (221). Knowledge of host-pathogen interactions in trees is limited and no critical studies have been conducted in palms. Woody plants are constantly exposed to potential invasive fungal species. Plant response to fungal attack determines the consequences of the interaction; whether the invader can invasively colonise the tissue or is repelled. Microorganisms potentially pathogenic to trees can be subdivided into six broad groups – wood decay fungi, canker rots, and endophytes, vascular wilts, xylem-invading bacteria and viruses (164). Active pathogens are able to colonise healthy sapwood following either direct penetration or infection through wounds and cause rapid spreading necrotic lesions such as *Heterobasidion* and *Armillaria* or systemic vascular invaders such as *Ophiostoma novo-ulmi* (181), whereas opportunistic fungi are less clearly aggressive and occupy functionally compromised or senescing tissue.

There have been various models proposed to explain the host-pathogen interactions in the wood of living trees. Boddy and Rayner (29, 181) proposed a model where the

ingress of a pathogen is not limited by host defences but rather that water potential and O_2 tension in functional sapwood itself is sufficient to preclude fungal development. According to this model, barrier layer/reaction zones have a wound repair function, serving to limit the spread of drying and cavitation and to maintain the hydraulic integrity of adjacent functional xylem. However, Shigo and co-workers (206) proposed the compartmentalisation of decay in trees (CODIT) model where static barriers are laid down preventing further spread of infection. A similar model describing a reaction zone model with a region of active host response at a dynamic interface between living sapwood and wood colonised by the pathogen was proposed by Shain (204).

A system that incorporates aspects from the earlier models was suggested by Pearce (164). A column barrier layer (CBL) reaction zone model was proposed where the reaction zone is a static barrier that serves to restrict or slow fungal ingress through a combination of factors including deposition of phytoalexin-like compounds such as phenolics and polyphenolics as well as microenvironmental factors. Pearce suggests the CBL regions and compartmentalisation may also have a wound repair function.

3.1.7.1 Formation of Structural Barriers

A general response to fungal infection of plants is formation of lignin and lignin-related compounds such as suberin, in the vicinity of invading hyphae. Tissue layers are also variable, and thickening of the cell wall or deposition of more recalcitrant polymers makes them more resistant to decay. In the primary tissues of both woody and herbaceous plants, cell wall appositions are commonly observed (164). These may incorporate a number of components not normally present in unmodified walls, including lignin and other phenolic compounds, callose and silicon together with normal cell wall material (10). Induced lignification may not be expected in already highly lignified xylem, however in the wood of *Hevea brasiliensis* taproots close to the infection front of *Rigidoporus lignosus* there was an increase in lignification of 25-30% (83). The main induced polymer associated with plant-pathogen interactions in xylem tissue is suberin (164). Suberin is not a typical polymer of woody tissue and is often induced in response to infection; its hydrophobicity and resistance to microbial degradation may play a role in compartmentalisation of infections. Suberisation of tissue could also act as a waterproof seal, reducing dehydration and

entry of air (30); also tyloses which develop from xylem parenchyma to occlude lumens of xylem vessels are commonly suberised (165).

3.1.7.2 Induced Antimicrobial Compounds (Phytoalexins)

A notable active resistance mechanism in woody plants is mediated by a non-specific response that appears to involve the formation of tissues resistant to fungal attack, but also the formation of phytoalexins (138). Phytoalexin-like compounds accumulating in the reaction zone of infected woody tissue is considered to retard fungal ingress and a glossary of these compounds found in the sapwood of trees were listed in the review by Pearce (164). These included phenols, lignans, phenylpropanoids, flavanoids, terpenoids and alkaloids. Flavanoids and polyphenols have widespread ability to inhibit spore germination of plant pathogens (94) and have been observed in the stem wood of *Erythrina latissima* and other trees (39). The isoflavin maackiain (3-hydroxyl-8,9-methylenedioxypterocarpan) is well known as a constitutive antifungal agent (phytoanticipin) in heartwood of legume trees and as an inducible phytoalexin in herbaceous legumes. Stevenson and Haware (214) showed that species of *Cicer bijugum* (wild chickpea) resistant to *Botrytis cinerea* contained 200-300 $\mu g\ g^{-1}$ of maackiain in foliage compared with less than 70 $\mu g\ g^{-1}$ in susceptible *Cicer* sp. When challenged with *B. cinerea*, concentration of maackiain increased to over 400 $\mu g\ g^{-1}$ in resistant species (214).

However, little research has been done to elucidate the range and function of induced antimicrobial compounds in the reaction zone of infected wood tissue and antimicrobial activity of some compounds has been low, suggesting that they may not be active phytoalexins. A recent study showed that crude wound extracts from *Eucalyptus* spp. were inhibitory to *Cladosporium cladosporoides*, *Ganoderma weberianum* and *Trametes zonata*. High Performance Liquid Chromatography (HPLC) and electrospray mass spectroscopy showed that formylated phloroglucinol compounds (FPC), and other polyphenolic compounds, were present in elevated quantities compared with sapwood, however bioassays containing purified FPC did not show antimicrobial properties (72). Although crude extracts obtained from wound wood demonstrate antimicrobial activity, it was recently proposed that the diverse range of metabolites formed might have multiple roles. For example, Eyles *et al* reported antioxidant activity of polyphenolic and terpenoid-based compounds released

by *Eucalyptus globulus* in wound extractives. They postulated that when released in response to wounding, they scavenge free radicals including superoxide and hydroxyl radicals, which are produced during wounding and cause damage to DNA, proteins and lipids (71), and therefore play a role in wound repair.

3.1.7.3 Microenvironmental Factors

In living wood, high water content of healthy sapwood is proposed to be a major factor in restricting fungal infections (29, 30, 181). High water content results in associated low O_2 content and elevated CO_2 concentrations (181), which can inhibit growth of wood decay fungi; however, many wood decay fungi are resistant to extreme O_2 and CO_2 levels (103). If the water content is high and mobile, this may remove extracellular fungal enzymes from the vicinity of the growing hypha and thus interrupt cell wall breakdown and metabolism, thereby impeding rapid decay of the woody tissue (30). In heavily decayed tissue, water levels (and O_2/CO_2 levels) are highly variable as they are often in direct contact with the air and isolated from the remainder of the xylem by the CBL (167). Pearce (164) suggests that many polyphenolics in the reaction zone may have a wound response function; the hydrophobic nature of these compounds may serve to limit the spread of decay by maintaining hydraulic integrity of the adjacent functional xylem, rather than having active antimicrobial activity. However, although antimicrobial activity of purified phytoalexin-like compounds found in wood tissue is sometimes low, the conditions *in vitro* do not match those within infected trees. When a potential pathogen may be exposed to many such compounds, perhaps acting synergistically or additively and therefore may be more active under natural conditions.

3.1.8 Fungal Pathogenicity Factors

To initiate infection, a pathogen must overcome the physical and chemical barriers set up by the host to block infection. Few studies have been conducted to determine what factors are involved with fungal pathogenicity in woody plants and none have been conducted in oil palm. Studies to elucidate virulence determinants from pathogenic fungi have often found that targeted mutation of suspected pathogenicity factors such as cell wall degrading enzymes have little impact on virulence. This has been attributed to functional redundancy of key pathogenicity genes within fungi. This has

been demonstrated in a recent study by Karlsson *et al* (111), where a cDNA library was constructed from total RNA extracted from *H. annosum* mycelium challenged with scots pine seedlings. Analysis of 923 expressed sequence tags (EST) produced by sequencing a random sample of cDNA showed that many of the genes expressed during early infection were involved in metabolism and protein synthesis, however numerous potential pathogenicity factors including glucanase and superoxide dismutase (SOD) were present in multiple isoforms. Redundancy of genes within fungi is also clearly shown from the genome of the basidiomycete *Phanerochaete chrysosporium*, which has ten genes encoding LiP, five encoding MnP and over two hundred and forty carbohydrate active enzymes (136).

No studies have so far been undertaken to determine how *G. boninense* degrades oil palm wood or events during infection. This chapter attempts to determine the enzymes produced by *G. boninense* during oil palm degradation and the effect these have on palm cell walls. Analysis of the process of infection can provide clues as to what factors are important for pathogenicity and increase understanding of the disease process. Additionally, development of a rapid method of infection using a small inoculum source may provide a means to allow plant breeders to screen for resistance, which has so far proved elusive.

3.2 Materials and Methods

3.2.1 Biochemical Analysis of Oil Palm Cell Wall Polymers

Oil palm wood was obtained from mature freshly felled commercial Tenera palms in Sumatra. Palm wood was shredded into 20x10x5 cm strips or cut into 12x6x6 cm blocks and dried for 2 h in a drying cabinet at 60°C. The wood was then packed into unsealed heat-resistant plastic bags and autoclaved at 121°C for 45 min. Samples were then re-dried in a drying cabinet overnight at 60°C before sealing in sterilised plastic bags to be sent to the UK. All wood component biochemical tests were carried out at the University of Bath, as described by Fry unless stated (80).

3.2.1.1 Alcohol Insoluble Residue (AIR)

Alcohol Insoluble Residue (AIR) was obtained by grinding wood samples in a Waring blender before adding ice-cold 70% w/v ethanol and stirring (magnetic stirrer) at 0°C for several hours. The resultant AIR was then collected by filtration through sintered glass; low molecular weight compounds such as sugars are removed in the filtrate. AIR was then subjected to extraction with ethanol:toluene (1:1) in a soxhlet for 25 cycles as described by (64) to remove low molecular weight aromatic and hydrophobic compounds. The sample was then washed in ethanol followed by distilled water until the filtrate no longer absorbed at A_{280nm}, before drying to constant weight at 70°C.

3.2.1.2 Cellulose Extraction

Twenty milligrams of extracted sample was treated with 3 ml acetic-nitric reagent (acetic acid:water:nitric acid (8:2:1)) and placed in a boiling water bath for 30 min to hydrolyse non-cellulosic polysaccharides. The sample was then cooled before centrifugation at 2,500 x g for 5 min and the supernatant discarded. Ten millilitres distilled water was added, re-centrifuged and the supernatant discarded. Ten millilitres of acetone was finally added, centrifuged and supernatant discarded as before. The residue was then left to dry in a desiccator overnight. The pellet was re-dissolved in 1 ml 67% H_2SO_4 and shaken for 1 h to hydrolyse cellulose and yield glucose, glucose was then assayed spectrophotometrically using the anthone assay for hexose sugars.

Anthrone assay. Approximately 10 µl of sample was added to a clean test tube and made up to 0.5 ml with distilled water. One ml 0.2% anthrone in conc H_2SO_4 was then added and incubated in a boiling water bath for 5 min, cooled and read at A_{620nm}. A glucose concentration standard was prepared and used for quantification of hoxose sugars using the anthrone assay (Appendix, Fig. 5).

3.2.1.3 Lignin Extraction

Klason lignin was obtained using the method described by Effland (64). Three hundred milligrams of the extracted sample was mixed with 3 ml 70% H_2SO_4 for 1 h at room temperature. Twenty-eight millilitres of distilled water was then added to the mixture/ml of H_2SO_4 and autoclaved for 1 h at 121°C for secondary hydrolysis. Lignin was recovered by filtration though a sintered glass Gooch crucible (Fisher) whilst still hot and washed with hot water to remove traces of acid. The crucible containing lignin was then dried in an oven at 70°C to constant weight.

3.2.1.4 Hemicellulose Extraction

After extraction of pectin the sample was treated with 0.15% acidified sodium chlorite (adjusted to pH 3.4 with acetic acid) at 60°C for 15 min in a fume cupboard (caution: toxic and explosive gas released). The sample was filtered though sintered glass and washed with water. Wood was then treated with 6 M NaOH (+1%$NaBH_4$) at 37°C overnight and then filtered through sintered glass to yield hemicellulose. The solution was then neutralised by slow addition of acetic acid before assaying hexoses with anthrone (as above) and pentoses using the orcinol assay.

Orcinol assay. To 0.5 ml solution (containing 1-10 µg pentose) 67 µl of reagent A (6% orcinol in ethanol) was added followed by 1 ml reagent B (0.1% $FeCl_3.6H_2O$ in conc HCl) and mixed. This was incubated for 20 min in a boiling water bath, cooled, mixed again and read at A_{665nm}. A xylose concentration standard was prepared and used for quantification of pentose sugars using the orcinol assay (Appendix, Fig. 6).

3.2.1.5 Starch Extraction

AIR was treated with 90% w/v dimethyl sulphoxide (DMSO) overnight at 25°C to solubilise starch and filtered on a sintered glass funnel. The filtrate was then treated with 5 vol of acetone at 0°C for several hours to precipitate starch. The filtrate was

centrifuged at 5000 x rpm for 5 min and the supernatant discarded. The pellet was resuspended in acetone and re-centrifuged, the supernatant was discarded and the pellet dried at 28°C overnight. Starch was then redissolved in hot distilled water before assay with anthrone at A_{620nm} (as above).

3.2.1.6 Pectin Extraction

Once treated with DMSO for starch (as above), the wood sample was then autoclaved at 121°C in 50 ml pyridine: acetic acid: water (PyAW) (1:1:23) for 30 min in a tightly screw capped bottle. The sample was filtered through a sintered glass funnel and rinsed with 2 x 50 ml SDW water in fume cupboard. Excess water and pyridine was evaporated in a rotary evaporator before freeze-drying to yield pectin. Pectin was then assessed gravimetrically.

3.2.2 Liquid Culture Conditions

G. boninense isolates BLRS 1 and R3 were grown in static liquid culture in the dark at 28°C in 250 ml Nunclon tissue culture flasks (Fisher) fitted with cap filters for aeration. Growth medium comprised 40 ml modified basal medium (see below) and 2g/10 ml dry weight of perlite to create semi-solid culture conditions. Basal medium was further supplemented with 0.05 g/l glucose as an initial energy source and 1.5% w/v oil palm cell walls (see below) to induce production of cell wall degrading enzymes.

3.2.2.1 Modified Basal Medium:

Basal medium modified from (47) was made up in 9.76g/l MES 2-N-morpholino ethanesulphonic acid 50 mM pH to 5.5 with NaOH. Basal medium consisted of 0.9g $(NH_4)H_2PO_4$, 2.0 g $(NH_4)_2H\ PO_4$, 1.0 g KH_2PO_4, and 0.5 g $MgSO_4.(7H_2O)$. Trace elements were made into a 100 x stock solution and consisted of 0.2 ppm $FeSO_4.7H_2O$, 1.0 ppm $ZnSO_4.7H_2O$, 0.02 ppm $NaMoO_4.2H_2O$, 0.02 ppm $CuSO_4.5H_2O$, 0.02 ppm $MnCl_2.4H_2O$ (Fisher).

3.2.2.2 Cell Wall Preparation for Liquid Cultures

Oil palm wood was reduced to small fragments in the UK and placed into a Waring blender to produce a fine sawdust-like sample. This was then soaked in excess potassium phosphate buffer (pH 6) before comminution by the blender at high speed

for 2-3 min. The resulting suspension was filtered through two layers of muslin and the insoluble plant material was resuspended in phosphate buffer for 5 min before again mixing in the blender. This was repeated for a total of four washes in the buffer and one wash in cold distilled water. Solvent extractions were performed in four washes (1:1 v/v) of chloroform:methanol (1:1 v/v) and three washes of 2 volumes of acetone. Resultant cell wall material was then left to dry overnight at 70°C in a drying oven before aseptic transfer to sterile glass jars. Jars were stored desiccated at room temperature with silica to prevent rehydration.

3.2.3 Enzyme Analysis

Enzyme activities were recorded from culture supernatant from liquid cultures or from enzyme solutions extracted from solid oil palm blocks.

3.2.3.1 Extraction of Cell Wall Degrading Enzymes (CWDE) from Wood Blocks

Oil palm wood blocks (3x3x3cm) were autoclaved at 121°C for 1 h and then inoculated with three 1 cm^2 discs of *Ganoderma* grown on PDA. *G. boninense* was then grown in the dark at 28°C for periods of 3 and 6 wks before enzyme extraction. Enzymes were retrieved from the wood blocks in an extraction buffer designed to prevent denaturation of the enzymes and to allow maximum desorption from the wood substrate (47). The buffer consisted of 5 mM dithiothreitol (to prevent oxidation), 0.2 M KCL (allowing desorption of proteins from cell walls) and 5% polyvinyl polypyrrolidone (to adsorb phenols) in 50 mM sodium phosphate [pH 6.0] as described by Williams *et al* (252) (all chemicals, Sigma, UK).

3.2.3.2 Dialysis of Culture Supernatant

Twenty millilitres of supernatant was filtered through sterilised muslin into dialysis tubing. Tubes were then sealed and placed in 10 mM MES buffer in a 5 l vessel in the cold room (4°C) overnight with stirring. The buffer was then changed and left for several more hours to remove low molecular weight sugars that contaminate reducing sugar enzyme assays. Supernatant was then re-concentrated to 20 ml using 35,000mw polyethyleneglycol (PEG) (FULKA). Half of the supernatant was then frozen and stored at –70°C, and a 10 ml working stock was used for immediate reactions and was stored on ice at 4°C.

3.2.3.3 Cellulase and Xylanase Assay (Nelson Somoygi)

Cellulases and xylanases were assayed by following the release of reducing sugars from the relevant substrate according to Somoygi's procedure (155). The substrates 0.1% birch wood xylan (Sigma, UK) and 1% w/v cellulose in the form of insoluble filter paper (Whatman No.1) were made up in 50 mM citrate buffer pH5.0. Reaction mixtures contained 50 µl enzyme test solution and 250 µl of substrate and were incubated at 37°C for 30 min (1 h for cellulase). Reactions were performed in duplicate with boiled controls used to measure background sugars. Reactions were stopped by transferring reaction tubes into a boiling water bath and adding 250 µl Somogyi's reagents A and B (Stop reagent was a mixture of reagents A and B at 25:1 prepared 30 min before use. Reagent A: g/l Na_2CO_3 25g, sodium potassium tartrate 25g, $NaHCO_3$ 20g, filtered. Reagent B: 15% $CuSO_4.5H_2O$ with 1 or 2 drops of H_2SO_4 added per 100 ml). Tubes were then allowed to cool to room temperature before addition of 250 µl of Somogyi reagent C (25 g of ammonium molybdate was dissolved in 450 ml of distilled water in clean acid washed glass bottle before addition of 21 ml of conc. H_2SO_4. Three grams of $Na_2HASO_4.7H_2O$ was dissolved in 25 ml of distilled water before addition to the ammonium molybdate solution. The reagent was then incubated in the dark at 37°C for 24 h). Tubes were then vortexed to mix and left for at least 2 h for the colour to develop. 200 µl was then removed to a microtitre plate and read at A_{595nm} against citrate buffer blanks. If necessary, samples were centrifuged at 11,600 x g for 10 min to remove undigested substrate before reading the supernatant. Concentration standards were made for glucose, xylose and galacturonic acid respectively (Appendix, Fig. 8-10), for quantification of products of enzymatic breakdown.

3.2.3.4 Glycosidase Assay

Activity was assayed by measuring increase at A_{410nm} from the release of *p*-nitrophenol from their respective *p*-nitrophenol linked substrates (β-D-galactoside and β-D-glucoside) as described (139). Glycosides were dissolved in 50 mM citrate buffer at pH5.0 at 5 mM. Each reaction mixture consisted of 180 µl of buffered glycoside solution and 20 µl of culture supernatant or boiled control. Reactions were performed in duplicate with boiled controls and buffer blanks. Reactions were stopped after incubation at 37°C for 30 min by addition of 500 µl 1 M $NaHCO_3$.

After 30 min the absorbance was read at 410 nm against citrate buffer blanks. A calibration curve was made for *p*-nitrophenol in the range 0-50 μM (Appendix, Fig. 11).

3.2.3.5 Remazol Brilliant Blue Xylanase Assay

Xylanase activity was assayed spectrophotometrically by measurement of enzyme-released dye fragments soluble in the presence of organic solvents which precipitate the original homogenic substrate, Remazol brilliant blue (RBB) dyed xylan (75). One hundred and eighty μg of 0.1% RBB dyed xylan in 50 mM citrate (pH5) was added to 20 μl culture supernatant, boiled enzyme or citrate blank. Reactions were incubated at 37°C for 30 min and then stopped by addition of 500 μl of 96% ethanol. Tubes were mixed and left to stand for 20 min before centrifugation at 11,600 x g for 2 min. 200 μl was then removed to a microtitre plate and read on a Dynatech MR500 plate reader against citrate blanks at A_{595nm}.

3.2.3.6 Pectin Lyase Assay

Pectin lyase activity was assayed spectrophotometrically by measuring the production of 4,5-unsaturated galacturonides at A_{240nm} in a Cecil spectrophotometer with quarz cuvettes, as described by Ayres (18). The reaction mixture contained 700 μl of 0.25% pectin in 50 mM Tris-HCl pH 9.0, 1 mM $CaCl_2$ (final concentration) and 300 μl of culture supernatant. The reaction mixture was incubated at 35°C and absorbance measured at time zero, 15 and 30 min for test (3 reps) and boiled controls for each sample. The molar extinction coefficient for pectin (ε_{240nm} = 5500 M^{-1} cm^{-1}) allowed conversion of optical density into product.

3.2.3.7 Endo-polygalacturonase (PG) Assay

PG was assayed viscometrically in Technico 200 viscometer at 37°C, as described by Durands (60). Activity is given as relative viscometric units (RVU) defined as 1000 x the reciprocal of time (min) for a 50% decrease in relative viscosity of 8 ml of NAPP solution buffer at pH 5.0 (0.1 M citrate) and 2 ml enzyme solution.

3.2.3.8 α-Amylase Assay

Amylase was assayed using the 3,5-dinitrosalicylic acid method adapted from Morgan and Priest (148). Reagent A (starch substrate): consisted of 20 mM NaH_2PO_4 with 6.7 mM NaCl in 100 ml deionised water adjusted to pH 6.9 at 20°C. To this 1% (w/v)

soluble starch was added and dissolved by heating on a hot plate at 70°C. Reagent B (colour reagent) was made up by dissolving 12.0 g sodium potassium tartrate in 8.0 ml 2 M NaOH by heating on a hot plate before adding to 20 ml 96 mM 3,5-dinitrosalicylic acid solution and diluting to 40 ml. Reactions were performed at 35°C. 100 µl culture supernatant was added to 100 µl soluble starch, mixed by swirling and placed in a water bath at 35°C for 15 or 30 min. 100 µl of reagent B was then added, re-sealed and placed in a boiling water bath for exactly 15 min then cooled on ice to room temperature and diluted with 700 µl deionised water before reading at A_{540nm}. A maltose concentration standard was used for quantification of maltose released (Appendix, Fig. 12) and boiled controls were used to determine background.

3.2.3.9 Lignin Peroxidase Assays

Azure B

Lignin peroxidase activity was measured at room temperature using the method described by Archibald (13). The reaction mixture contained 32 µM Azure B and 100 µM H_2O_2 in 50 mM Na tartrate buffer (pH 4.5, 25°C). The dye and H_2O_2 were made up in x100 stock solutions and 10 µl of each was added to a 1 ml final reaction volume, 500 µl of culture supernatant (or enzyme extraction buffer) was used for each reaction and assays begun with addition of H_2O_2. Decrease at A_{651} was determined over a 15 min period using a chart recorder. Activity was determined by referring to a concentration standard for Azure B at A_{651nm} (Appendix, Fig. 13). Reactions were performed in duplicate with boiled controls.

Veratryl Alcohol

Assessment of lignin peroxidase activity was also attempted spectrophotometrically by measurement of oxidation of veratryl alcohol to veratrylaldehyde (ε_{310nm} = 9,300 $M^{-1}cm^{-1}$) in the presence of H_2O_2, at room temperature as described by Tien and Kirk (228). Reaction mixtures had a total volume of 0.5 ml containing 335 µl of culture supernatant and 165 µl of 0.4 mM veratryl alcohol and 0.15 mM H_2O_2 in 100 mM sodium tartrate (pH 3). The reaction began with addition of H_2O_2 and the linear increase in absorbance was measured over a five-minute period at 310 nm. Reactions were performed in duplicate with boiled controls.

3.2.3.9 Laccase Assay (ABTS)

Laccase activity was measured using the protocol adapted from Yaropolov *et al* (256), which measures the oxidation of 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). Reaction mixtures contained 14 μM ABTS in 50 mM glycine-HCL buffer (pH 3.0, 30°C) in a 1 ml reaction mixture containing 0.5 ml culture supernatant. Reactions began with mixing the culture supernatant with the buffer containing ABTS in a cuvette. The cuvette was immediately placed in a spectrophotometer and read at A_{405nm} for 5 min. It was determined that the linear rate of reaction could be accurately calculated from absorbance readings recorded over 200 seconds (Appendix, Fig. 14). Reactions were performed in duplicate with boiled controls.

3.3.4 Oil Palm Seedlings

Oil palm seedlings used for pathogenicity studies were as described (See 2.2.3).

3.3.5 Preparation of Wood Blocks and Inoculation of Oil Palms

Rubber-wood blocks were used as a food source for infection of oil palm seedlings by *G. boninense*. Blocks were prepared as described (see 2.2.4).

3.2.6 Light Microscopy of Infected Oil Palm Roots

All light microscope staining was done prior to adding a cover slip to the slides. Unless stated separately all stains were made up as 1% aqueous solutions using distilled water.

3.2.6.1 Calcofluor white

Calcofluor white was obtained from Sigma and 50 mg added to 5 ml Tris-buffer (pH 9.0) buffer to make a stock solution. The working solution was obtained by dilution of 100 μl stock solution to 9.9 ml Tris buffer. The stain was applied directly to the sections on slides for about 1 min then washed off with distilled water and viewed under UV light. This fluorochrome stains 1,4-linked polymers (cellulose, chitin) and thus shows up hyphal and plant cell walls (40).

3.2.6.2 Sudan IV

Sudan IV (Fisher Scientific Ltd) shows up suberin and protein bound lipids very darkly and was used to assess the degree of suberization of root tissue.

3.2.6.3 Epoxy Tissue Stain

This stain contains Toluidine blue and basic fuchsin which show up areas of lignin well and is good for a general stain. Obtained from Electron Microscopy Sciences, Fort Washington, USA.

3.2.7 Transmission Electron Microscopy

Preparation of samples was conducted in a fume hood. Samples were collected and placed in 3.5% glutaraldehyde (Agar Scientific) in 0.05 M piperazine-*N*, *N* '-bis (2-ethanesulfonic acid) (PIPES) buffer at pH 8.0 to fix proteins. Whilst submerged, tissue was then cut into 1 mm^3 pieces and exposed to vacuum for 16-20 h to extract trapped air. Samples were then rinsed three times in 0.15M PIPES on a rotor for 15-20 min per rinse. Samples were then placed in 1% osmium tetroxide in 0.15 M PIPES buffer on a rotor for 1 h. Osmium was then removed by three 15 min washes in miliQ water. Dehydration of the samples was conducted sequentially in 30%, 50%, 60%, 70%, 80%, 90%, 95% and finally 100% acetone. Samples were left in each dilution for 30 min with three changes. Samples were then placed in propylene oxide (TAAB) on a rotor overnight. Infiltration of the samples begins with exposure to a mixture of 3:1 propylene oxide: SPURR resin (TAAB) for approximately 12 h on a rotor. The ratio is changed to 1:1 propylene oxide: SPURR resin overnight. The ratio is again changed and placed in a ratio of 1:3 propylene oxide: SPURR resin for a period of 2 h. 100% epoxy resin is finally added and changed three times over a 6 h period. The samples can then be left in 100% SPURR resin before embedding. Samples were placed in moulds and submerged in 100% SPURR resin. Moulds were then placed in a drying oven at 70°C for 7 h to allow the resin to set.

Ultra thin sections were then cut with a microtome diamond knife. Cut sections were stained using uranyl acetate and Reynolds lead citrate before viewing by transmission electron microscopy (JEOL TEM1200EX transmission electron microscope).

3.3 Results

3.3.1 Polymer Composition of Oil Palm

Cellulose content was determined using the anthrone assay for hexose sugars on the products of the cellulose extractions and hemicellulose extraction components were assayed using both orcinol for pentose sugars and anthrone for hexose sugars. When comparing mature and juvenile (seedling) oil palms the main differences in the polymer composition are in the starch and cellulose content (Fig 44a,b). The bole of oil palm seedlings had an extremely high starch content compared to mature palm stem tissue, 66% compared to 2% respectively, whereas the mature palm has a cellulose content of 56% compared with just 13% in the juvenile. Hemicellulose is also proportionately higher in mature oil palm trees, 18% compared with 7% in seedlings. Pectin content was unexpectedly lower in oil palm seedlings (2%) than in mature oil palm tissue (4%) as the middle lamella of seedlings usually contains a high proportion of pectin, which is subsequently replaced by lignin in mature tissue. However, the necessary proximity of tissue used for analysis to the meristem (due to stem size) means that cells may contain a lot of storage polymers that are not present in mature tissue and skew the data so that the importance of the pectin component of cell walls may be underestimated in the juvenile. Nevertheless, cell walls in mature oil palm are much more developed and thickened than in 18 month-old plants.

3.3.2 Cell Wall Analysis of Decaying Palm-wood Blocks

Analysis of polymer content of wood from a naturally infected, diseased tree showed a similar pattern of decay to oil palm wood blocks decayed by *Ganoderma in vitro* (Fig. 44c,d). Duration of decay in the naturally infected material was indeterminable and it was not possible to determine component dry weight loss from this material. However, overall proportions of the polymers are similar and starch was completely removed from both laboratory and naturally degraded tissue. Cellulose appeared to be attacked proportionately more in the natural tissue (46% cellulose remaining) than *in vitro* (53%). However it is not known if the proportion of cellulose from wood degraded *in vitro* and left for a longer period would approach that of the natural decay

sample. The proportion of lignin was similar in both naturally and *in vitro* degraded wood (18% and 21% respectively), this was substantially higher than control wood (11%) suggesting that lignin is not removed as readily as cellulose. Analysis of the pectin and hemicellulose content of decayed wood was complicated by presence of fungal cell wall polymers within the tissue. Hemicellulose and pectin could not be accurately quantified as the extraction processes also resulted in extraction of fungal cell wall polymers present in the degraded tissue (Fig. 44c,d).

Dry weight loss from the oil palm blocks occurred rapidly over the first 6 wks (17% after 3 wks and 50% after 6 wks). Little additional weight loss occurred after this time and the average weight loss of blocks degraded by *Ganoderma* after 9 wks was 54% (Fig. 45). Degradation of cellulose also followed this trend with 20%, 55% and 58% loss after 3, 6 and 9 wks respectively (Fig. 45). Lignin was degraded to a similar extent with percentage loss of 27%, 55% and 61% respectively after 3, 6 and 9 wks. Although accurate data for loss of hemicellulose and pectin could not be obtained, complete loss of starch from the wood blocks and a large reduction in both cellulose and lignin polymers suggests that *G. boninense* is a simultaneous degrader of oil palm wood. Though starch represents a minor component of mature oil palm wood (Fig. 45a.), complete loss of starch was observed from all reps within 3 wks.

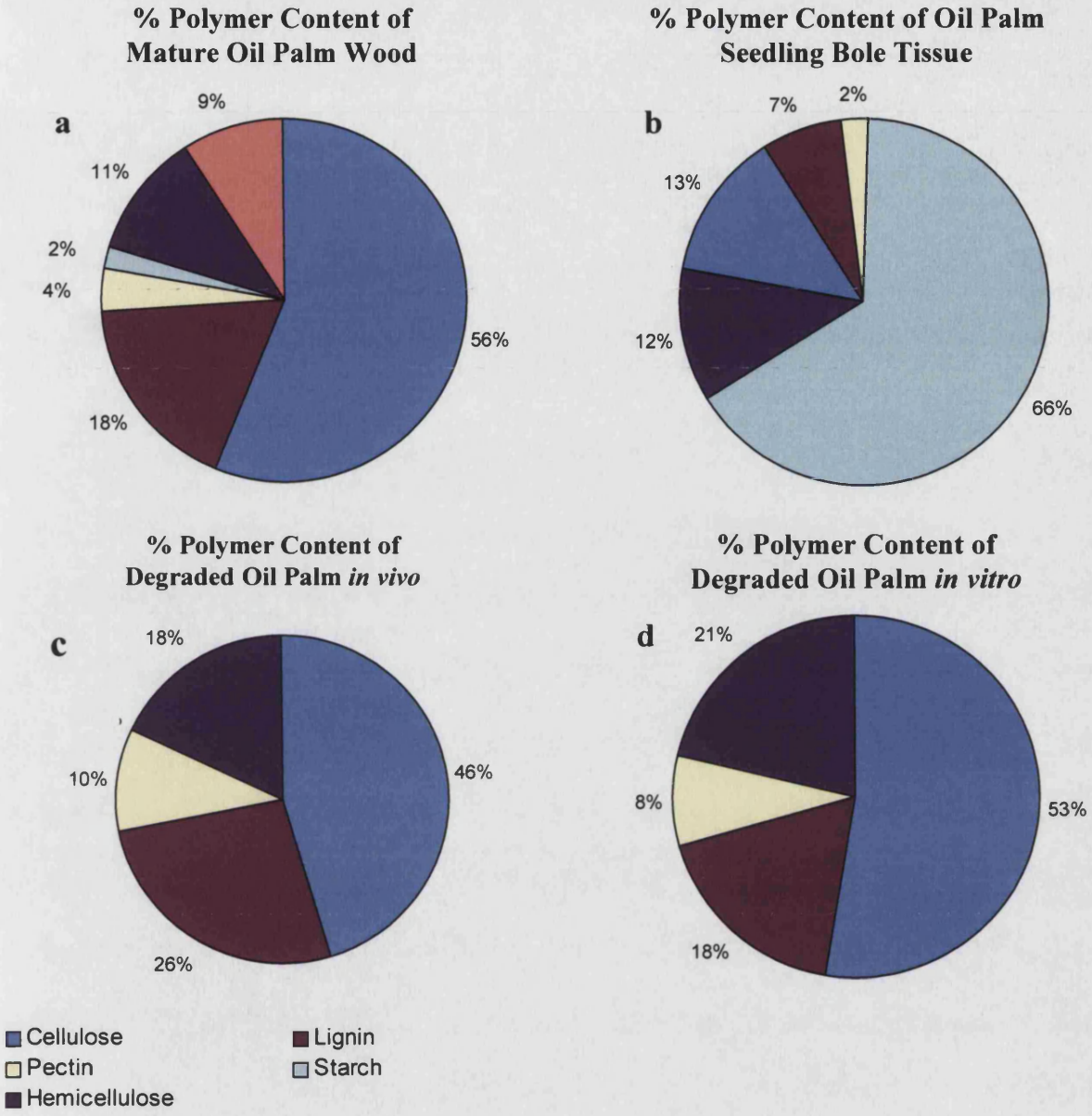

FIG. 44. **Percentage polymer composition of decayed and un-decayed oil palm tissue. a.** Polymer composition of un-degraded 25 yr oil palm trunk tissue. **b.** Polymer composition of un-degraded18 month oil palm seedling. **c.** Composition of decayed palm tissue from a felled plantation palm. **d.** Composition of oil palm wood blocks after 9 wks of decay under laboratory conditions.

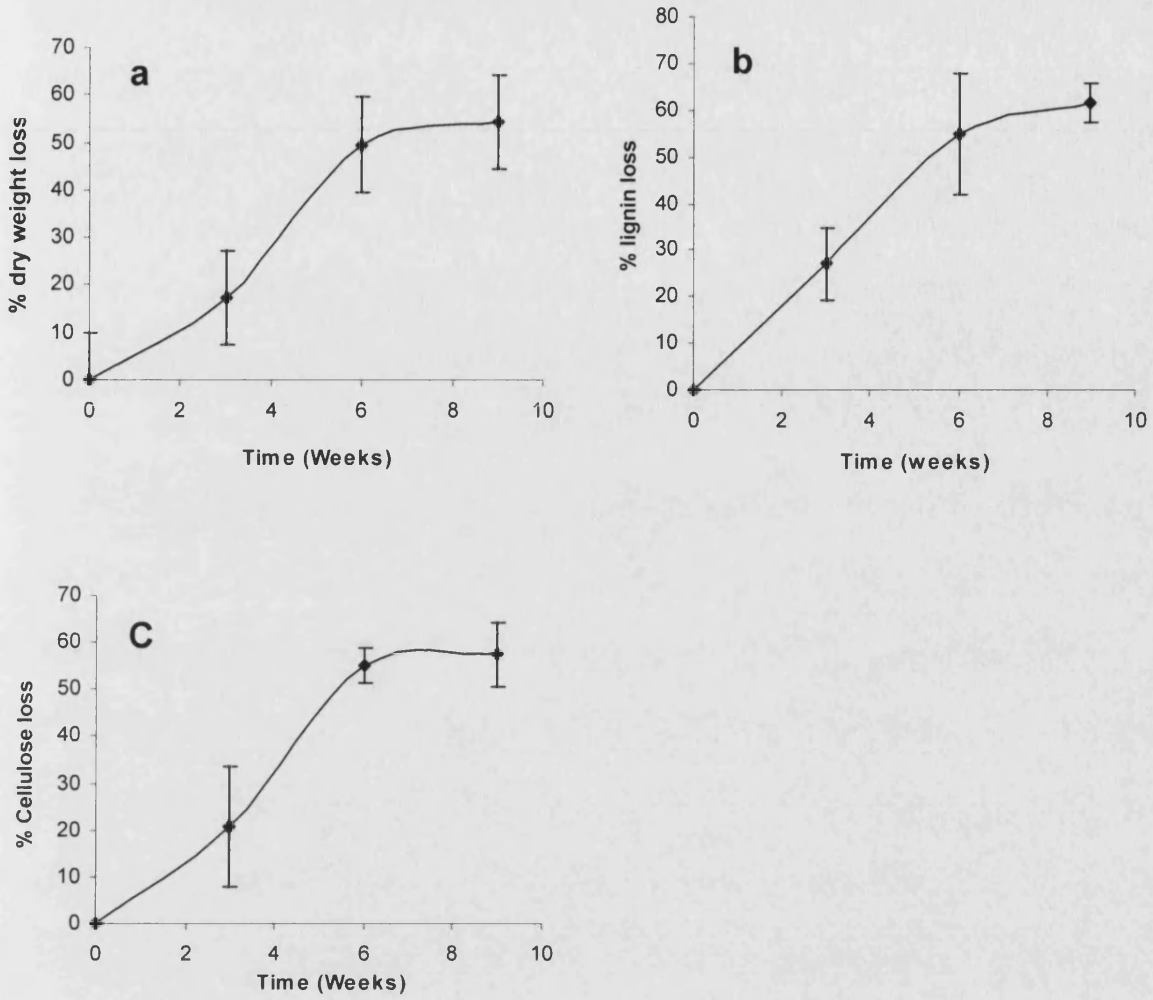

FIG. 45. Percentage dry weight, lignin and cellulose loss from oil palm blocks during 9 weeks colonisation by *G. boninense* BLRS1. **a.** Percentage dry weight loss of oil palm wood blocks. **b.** Percentage loss of lignin. **c.** Percentage loss of cellulose. Error bars indicate standard deviation of means of three replicates per time point.

3.3.3 *Ganoderma* Cell Wall Degrading Enzymes

Ganoderma boninense, as a white rot fungus is predicted to produce an array of cell wall degrading enzymes including ligninases. Analysis of wood decayed by *G. boninense* over 9 weeks provided evidence that *Ganoderma* could degrade cellulose, lignin and starch polymers, however accurate data for the other major plant polymers, pectin and hemicellulose, could not be obtained. Liquid cultures containing minimal medium and oil palm cell walls were established to try to stimulate production of cell wall degrading enzymes, to determine the array of enzymes produced and to quantify

enzymatic activity. Individual enzymes were not isolated or characterised at this stage, as the aim was to determine the potential of *G. boninense*.

Obtaining enzyme activity from liquid cultures was more difficult than anticipated as the nitrogen source in minimal medium had to be changed from nitrate to ammonium to enable sufficient growth of the pathogen. Furthermore, to encourage sufficient mycelial growth it was necessary to adapt the liquid culture by addition of perlite to create semi solid conditions, shown to enhance growth and enzyme production by other basidiomycetes (58, 242). Cellulose, lignin, hemicellulose, polygalacturonic acid (pectin) and starch are the most prevalent polymers in oil palm wood and enzymes were obtained that degrade most of these polymers.

However, despite the complete loss of starch in colonised wood blocks, no α-amylase activity was detected from the liquid cultures. *G. boninense* was grown in liquid culture supplemented with either AIR, insoluble starch or maltose to potentially stimulate production of starch degrading enzymes or supplemented with glucose as a negative control. Qualitatively, *G. boninense* grew well on AIR, insoluble starch and glucose, but did not produce a dense mycelium mat in cultures enriched with maltose. To determine if enzymes remained bound to cell walls, mycelia from each time point were washed with 0.2 M KCl in 50 mM sodium phosphate [pH 6.0] to release any enzymes that might remain bound to fungal cell walls. The samples were then dialysed before analysis, however, no α-amylase activity could be detected from these samples. Assays were also carried out on enzyme extractions from colonised wood blocks, but high background maltose levels, even after dialysis, meant that it was impossible to conduct accurate analysis.

From the liquid cultures cellulase activity was detected using the Nelson Somoygi reducing sugar assay (Fig. 46a.). Activity was first observed after 4 days and steadily increased throughout the time course. At the end of the sample period cellulase activity was continuing to increase, probably due to increasing amount of *Ganoderma* mycelium and exhaustion of alternative carbon sources. The assay quantifies the amount of reducing sugar produced from cellulose microfibres in filter paper and relates predominantly to the combined action of EG, CBH and β-glucosidase enzymes. Specific β-glucosidase activity, recorded using *p*-nitrophenyl glucoside,

showed higher activity than overall cellulase activity. This probably reflects the solubility of the model substrate compared with relatively inaccessible crystalline cellulose.

Xylanase activity was also readily obtained from liquid cultures using the Nelson Somoygi method and activity was confirmed using the RBB xylanase assay, which showed a similar time course. Xylanase activity was markedly greater than cellulase (maximum 120 nkats vs 9 nkats respectively), however birch-wood xylan is a more accessible molecule for degradation than crystalline cellulose. As with cellulose degradation, hemicellulose is degraded by the combined action of several enzymes such as 1,4-β-D-xylanases and acetyl xylan esterases. Therefore activities do not correspond to a particular enzyme but rather to the synergistic action of numerous xylan degrading enzymes.

Pectin degrading enzymes endopolygalacturonase (PG) and polygalacturonide lyase (pectin lyase, PGL) have similar activity profiles. In contrast with all other enzymes assayed, PGL activity was present after only 2 days and increased rapidly until it reached a peak of 200 nkats/ml after 10d (Fig. 47). After this time no PL activity was detected. Endopolygalacturonase also cleaves the α-1,4-linked pectin backbone and activity was assayed viscometrically (Fig. 47). Activity was present after 10 days and increased to a maximum after 15 days when activity began to decline. Reduction in pectinase enzyme activity from peak around 10-15 days, despite the increasing mycelial mass, suggests that pectin polymers had become limiting in the liquid cultures towards the end of the sample period. This is not surprising considering the low (4%) pectin content observed in oil palm wood tissue from chemical analysis.

The profile of pectic enzyme production confirms differential regulation with cellulases as production of these enzymes is still increasing by the end of the time course. Galactosidase activity remained high until the end of the sampling period (Fig. 47); this was probably as a result of galactose units on side chains in hemicellulose and other polysaccharide components of the cell walls, including pectins.

Lignin peroxidase (LiP) production was not stimulated under liquid culture conditions, despite analysis using several different assays reported to be effective for quantification of lignin peroxidase from liquid cultures of white-rot fungi (13, 228). Liquid cultures were supplemented by addition of tryptophan, veratryl alcohol and syringic acid, which have been shown to stimulate LiP in other fungi (44, 73, 125). It was, however, possible to quantify lignin peroxidase activity from degrading wood blocks by direct extraction of the enzymes from the solid substrate. Lignin peroxidase (LiP) activity from wood at the advancing edge of mycelium was similar to LiP activity from extensively colonised tissue near the point of inoculum from two isolates of *Ganoderma* (Fig 48). Activity was *ca.* 200-250 nkats/g fresh-weight of wood tissue for tissue from colonise blocks after three weeks for both. After 6 wks wood blocks were fully colonised and tissue was extensively degraded. Enzyme activity from isolate BLRS1 was much higher at this stage, 345 nkats, whereas SBJ1 activity similar to activity recorded after three weeks.

Unlike LiP, laccase activity was obtained from all liquid cultures and the appendix (Table. 3) shows mean activities from all treatments during the timecourse. Liquid cultures with oil palm cell walls as a carbon source were shown to induce greater laccase production. Strong activity was obtained from cell wall cultures supplemented with veratryl alcohol, tryptophan and syringic acid and activity profiles were comparable for each treatment (Fig. 50a&c). Glucose does not induce lignin-degrading enzymes, but supplementation of glucose cultures with veratryl alcohol, tryptophan and syringic, could potentially stimulate these enzymes. However, only veratryl alcohol stimulated laccase activities comparable with cell wall cultures. Indeed, the strongest activity profile was obtained from liquid cultures containing low nitrogen, glucose and veratryl alcohol in which maximum activity was 0.171 AU/min in these cultures after 4 days (compared with 0.133 AU/min in veratryl alcohol cultures with cell walls) and activities generally remained higher for the remainder of the time course. This suggests that veratryl alcohol may be used as a substitute for cell walls for future study of laccase production in *G. boninense*. Activities from tryptophan and syringic acid glucose cultures were negligible in the early stages (<0.014 AU/min for the first 8 days). Greater activities were observed after 13 days and induction of laccases from the later samples could coincide with nitrogen and carbon sources becoming limiting. High nitrogen may inhibit expression of lignin

degrading enzymes and in this study *G. boninense* basal medium liquid cultures contained either high (40 mM) or low (4.0 mM) nitrogen concentrations to investigate its affect on production. Laccase activity was generally greater under low nitrogen conditions. Activity from veratryl alcohol supplemented cultures under nitrogen limiting conditions was more than double the activity in high nitrogen after 4 days and remained higher throughout the experiment (Fig. 50a-d). Higher laccase activity was also generally observed from cultures with tryptophan and syringic acid under low nitrogen conditions compared with high nitrogen conditions. Extracellular laccase production by *G. boninense* was also detected on GSM solid media by the brown discoloration of the agar resulting from oxidation of tannic acid (Fig. 49).

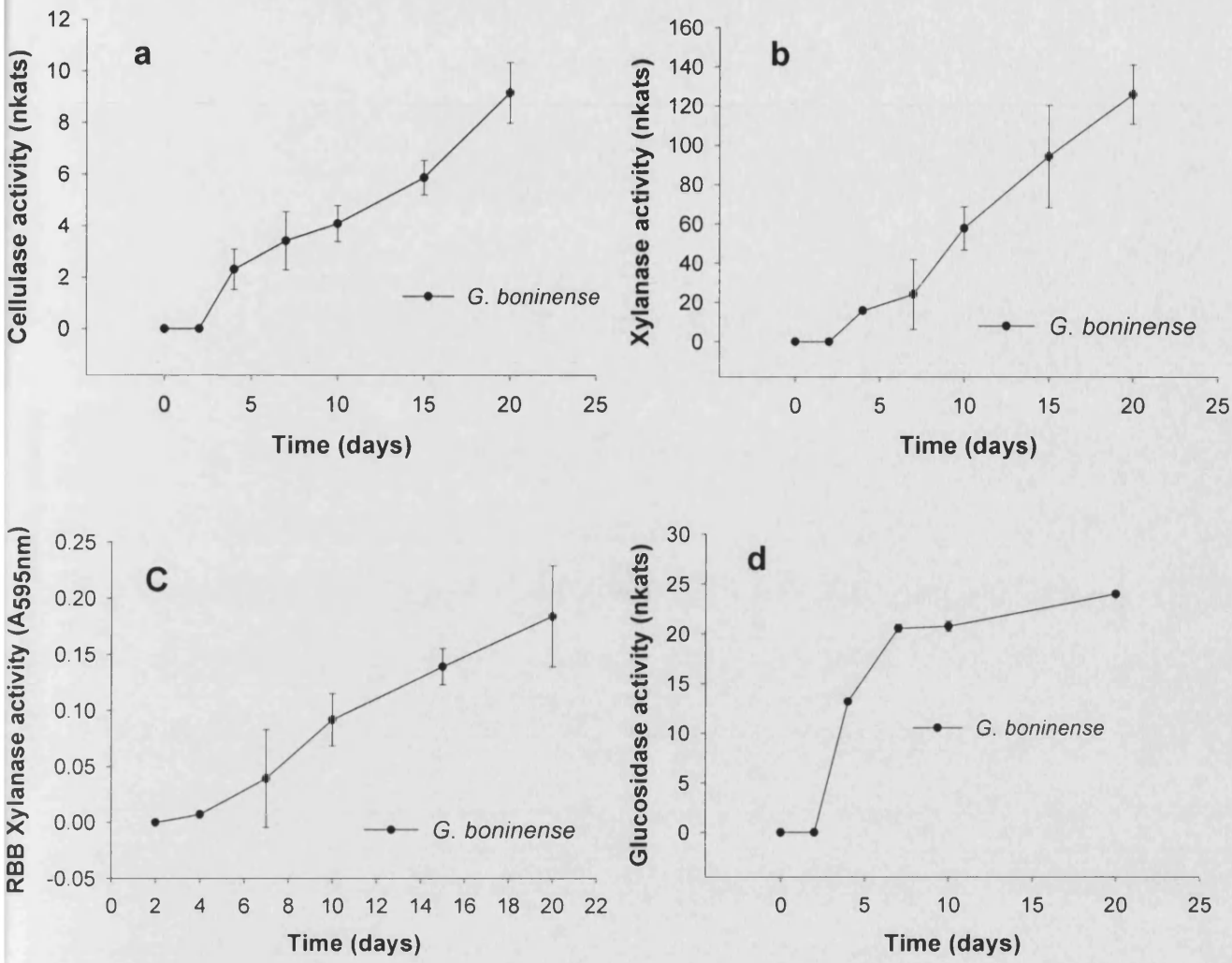

FIG. 46a-d. Activities of cellulase, xylanase and glucosidase enzymes from *G. boninense* liquid cultures. Activities are presented as nkats/ml culture supernatant except for RBB xylanase, which is shown as an increase A_{595nm} as a result of cleavage of a dye-linked polymer. **a**. Cellulase activity of *G. boninense* assayed by Nelson Somoygi reducing sugar assay. **b**. Xylanase activity using Nelson Somoygi assay. **c**. Xylanase activity measured by RBB xylanase. **d**. Glucosidase activity. Error bars indicate standard deviation of means of three replicates per time point.

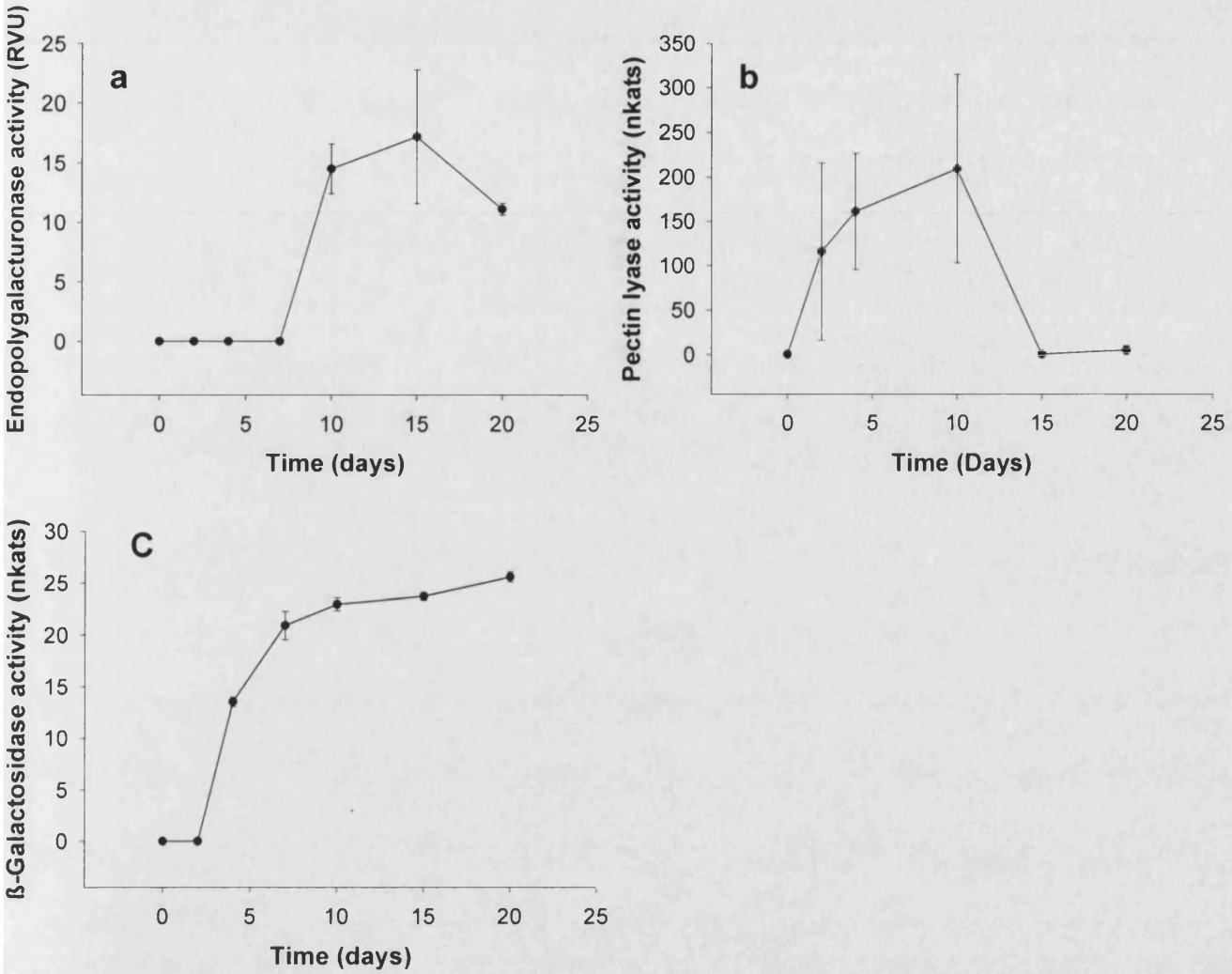

FIG. 47a-c. Activities of pectin degrading enzymes and galactosidase from *G. boninense* liquid cultures. Activities are presented as nkats/ml culture supernatant with the exception of endopolygalacturonase, this was assayed by viscometry and activity is presented as RVU. **a** Endopolygalacturonase (EG). **b** Pectin lyase (PGL) activity. **c** β-Galactosidase activity. Error bars indicate standard deviation of means of three replicates per time point.

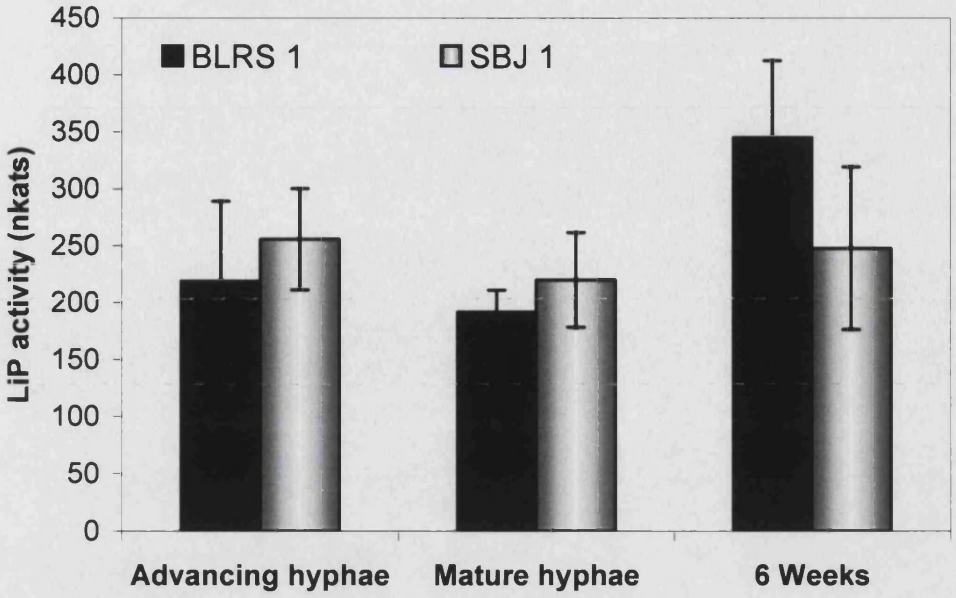

FIG. 48. Lignin peroxidase activity from degrading wood blocks by two isolates of *G. boninense*: BLRS1 and SBJ1. Wood tissue was taken from the advancing edge of mycelium (advancing hyphae) and from established colonised wood (mature hyphae) after 3 wks. All tissue was fully colonised after 6 wks and heavily degraded. Error bars indicate standard deviation of means from three replicates.

FIG. 49. Laccase production by *G. boninense* on GSM. Enzyme activity shown by brown discoloration of medium due to laccase oxidation of tannic acid.

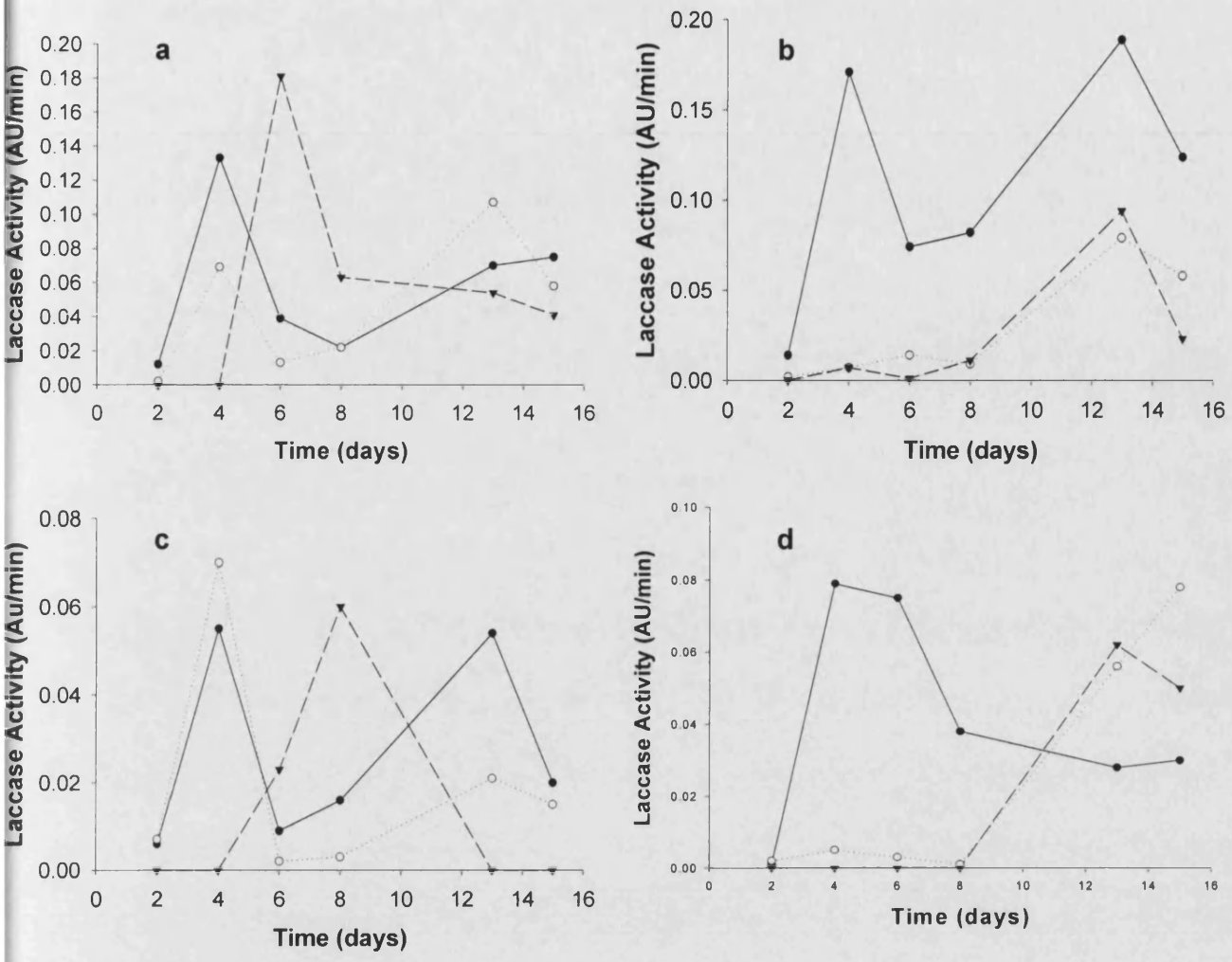

FIG. 50. Effect of nitrogen concentration and supplements on laccase activity in liquid cultures. Activity is expressed as absorbance units/min and values represent the mean of two replicates for each time point, for clarity, the standard deviation is tabulated in appendix, Table 3. **a.** Low nitrogen conditions with oil palm cell walls as a carbon source. **b.** Low nitrogen with glucose carbon source. **c.** High nitrogen with oil palm cell walls. **d.** High nitrogen with glucose. ● = veratryl alcohol supplement, ▼ = syringic acid, ○ = tryptophan.

3.3.4 Structure of Oil Palm Roots

Oil Palm root architecture and distribution as described by Jourdan and Rey (110) are similar to the palm *Serenoa repens* as described by Fisher and Jayachandran (76) and anatomy of palm roots are described in detail by Seubert (203). Oil palm roots are extremely tough and consist of several cell layers, which present a major barrier for disease establishment by root pathogens. The outer cell layer (epidermis or rhizodermis) of the oil palm is made up of large, thin-walled cells with no evidence of root hairs (Fig. 51a&c). Immediately below this cell layer is the exodermis. These cells stain brightly when exposed to calcofluor white demonstrating thickened secondary cell walls (Fig. 51a). The exodermis also stains darkly with Sudan IV showing high density of suberin and protein bound lipids. Thickened cell walls enriched with suberin must form a significant barrier to penetration by fungal pathogens, including *Ganoderma.* Below the exodermis is the outer cortex and this consists of at least two layers. The first layer is thin-walled; approximately three cells thick and does not stain with calcofluor or Sudan IV. The next layer is thick walled, is enriched with suberin and presents the next major obstacle for infection.

Beneath the barrier layers is the inner cortex; this layer does not have thickened secondary cell walls and its thin, primary cell walls may be easily degraded by pathogens. Throughout this layer there are multiple air spaces called 'lacunae' running longitudinally within the root, which could potentially facilitate rapid progression of the pathogen through the root. The barrier between the cortex and the vascular cylinder (stele) is called the endodermis. This is suberised but does not stain brightly with calcofluor (Fig. 51b). The parenchyma cells of the vascular cylinder stain brightly with calcofluor due to extremely thick secondary cell walls. Lacunae also run longitudinally through this tissue but are much smaller than those in the inner cortex (Fig. 51a-b).

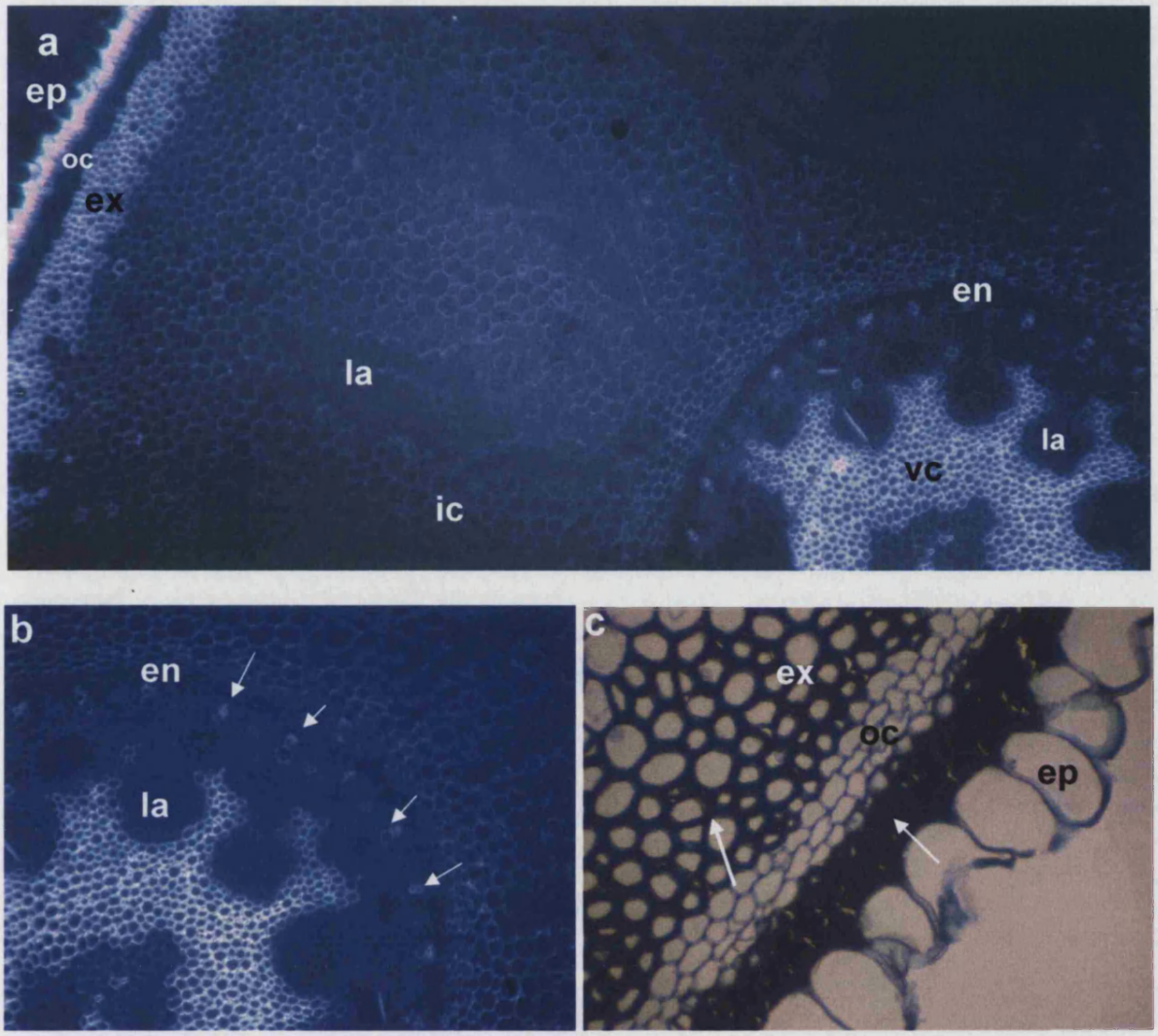

FIG. 51. Root anatomy of healthy oil palm roots. **a**. Cross section from oil palm root: epidermis/rhizodermis (ep), exodermis (ex), outer cortex (oc), inner cortex (ic), lacunae (la), endodermis (en) and vascular cylinder (vc) (x100 magnification). **b**. Higher magnification of vascular cylinder showing vascular bundles (arrows) arrayed around the perimeter, immediately below the endodermis (x400 magnification). **a-b**. Sections were treated with calcofluor white to show up thickened secondary cell walls. Under UV light, bright white areas indicate thickened secondary cell walls; the epidermis, exodermis and most of the vascular cylinder therefore have thickened secondary cell walls. **c**. Section is stained with Sudan IV. Darkly staining regions indicate cell walls densely enriched with suberin (arrows) (x1000 magnification).

3.3.5 *Ganoderma* Infection of Oil Palm Roots

Artificial inoculation of non-wounded roots was conducted using colonised rubber-wood blocks (See chapter 1). Fusion of the inoculum source to the surface preceded penetration into the root and infection. Mycelium degraded the outer barrier layers and this was followed by penetration of hyphae into the more easily degraded inner cortex. *Ganoderma* then progresses throughout the inner cortex, extensively

degrading primary cell walls, resulting in complete destruction of the inner cortex (Fig. 52a.).

Cells of the cortex were invaded by numerous hyphae (Fig. 52b.) which probably first utilise host cellular components before secreting cell wall degrading enzymes to colonise further and to utilise host cell wall polymers. Transmission electron micrographs reveal in detail extensive breakdown of cortical cell walls (Fig. 52c-d). The cell wall is attacked in multiple localised areas by the pathogen, suggestive of simultaneous degradation of cell wall components. The secreted enzymes attack all cell wall layers, ultimately resulting in complete breakdown of the cell wall including the middle lamella, facilitating fungal passage between cells.

There was no evidence of the fungus using lacunae to facilitate rapid progress through the host; instead the fungus appeared to progress from cell to cell along the root, mainly within the cortex where the thin primary cell walls offer little resistance. Despite the ability of *G. boninense* to penetrate the barrier layers (exodermis/outer cortex) as a pre-requisite for infection, the recalcitrant nature of these tissue layers mean that progress of root colonisation occurs mainly through the inner cortex. Similarly, the robust nature of the endodermis and vascular cylinder mean that heavily infected roots often have intact outer cell layers disconnected from an undegraded stele. Eventually even the thickened cell walls of the outer cortex and the vascular cylinder are also destroyed and remaining root fragments disintegrate in the soil.

3.3.6 Stroma Formation

Where a large inoculum source is present, mycelia may grow from the food-source around the root and grow partially along the surface (Fig. 53a). This fungal mass becomes encrusted and pigmented with melanin. Transmission electron micrographs of this material show that fungal cell walls become extensively thickened and pigmented. Some of the cells lose their cytoplasm and become metabolically inactive. However, some of the cells do not have such thick cell walls and remain metabolically active (Fig. 53b&c) and this is supported by the ability to re-isolate *G. boninense* from stroma (data not shown).

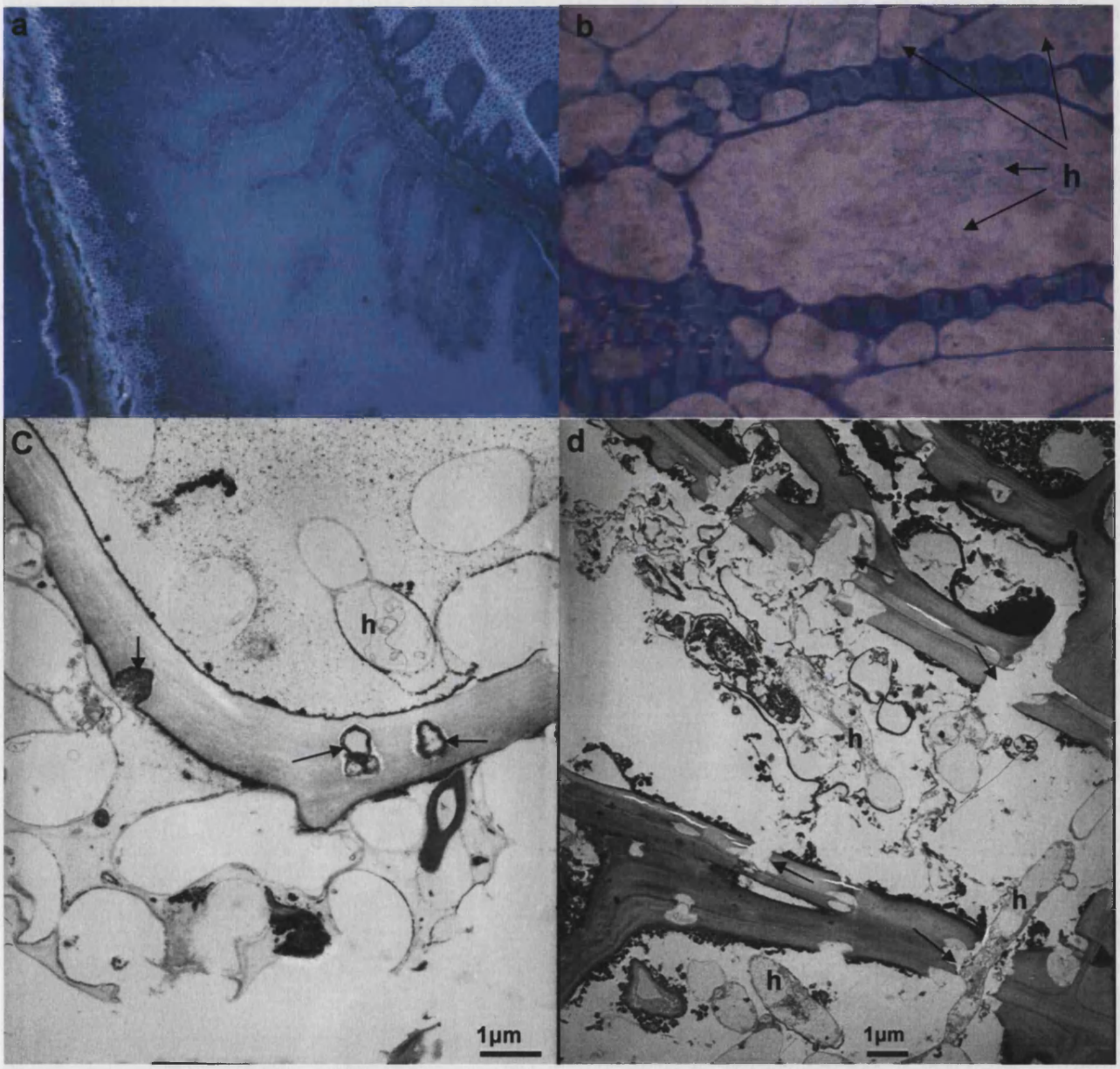

FIG. 52. Light and Transmission electron microscopy of infected oil palm roots. a. UV light micrograph of severely degraded oil palm root stained with calcofluor white. Outer cortex, exodermis and epidermis are disorganised but cells have not yet been fully degraded. Endodermis and the vascular cylinder do not show obvious signs of disruption. The inner cortex is almost completely destroyed and organisation of the lacunae has been degraded (x100 magnification). **b**. Light micrograph, stained with toluidine blue and basic fuchsin, shows cortical cells entirely colonised by thick *G. boninense* (x1000 magnification). **c-d**. TEM micrographs showing intracellular hyphae (h) and enzymatic decay of cortical cell walls (arrows). Note the discreet nature of the decay regions and that the degradation is not confined to any particular cell wall layer.

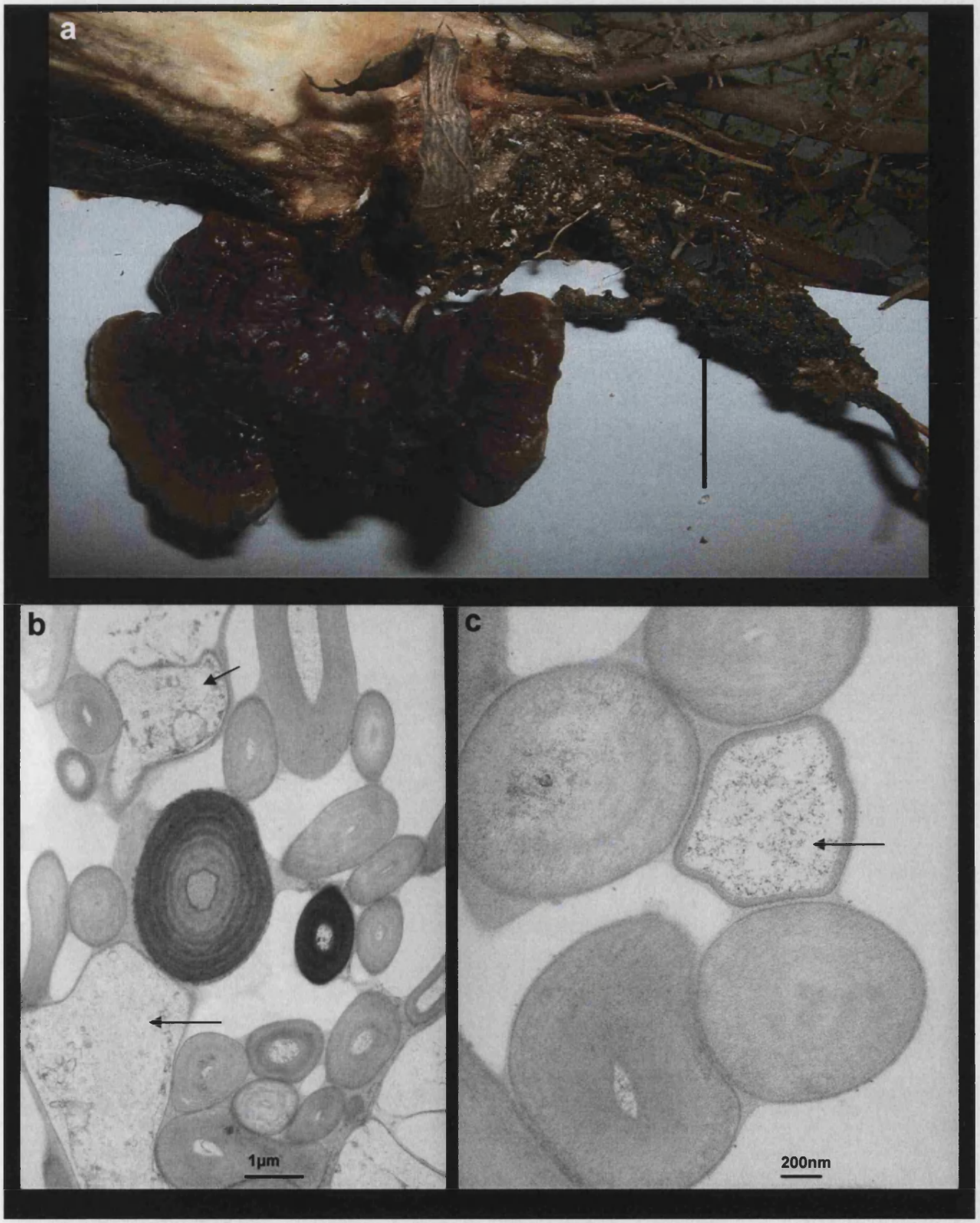

FIG. 53. Macroscopic and microscopic images of stroma from *G. boninense* during infection. **a**. Infected root showing stroma growing over the surface of the root and towards the base of the palm (arrow). A basidiophore has also developed on the stem base. **b**. Transmission electron micrograph of stroma cells. Note the extremely thick, melanised, cell walls that have all but lost their cytoplasm. Some cells however remain metabolically active (arrows) and are perhaps protected by the neighbouring melanised and thickened hyphae. **c**. Higher magnification of stroma cells showing hyphae almost devoid of cytoplasm and consisting simply of cell walls and a metabolically active hypha with only slightly thickened walls.

3.3.7 Infection of Basal Stem Tissue: Yellow Zone

The parenchyma cells found at the base of a juvenile oil palm are typically filled with multiple starch grains (Fig. 54a-b.). The cells also have a large vacuole occupying most of the cell with the cytoplasm appressed to the cell wall. Once *Ganoderma* reaches the base of the oil palm the typical infection pattern results in a 'yellow zone' appearing immediately in front of the 'reaction zone', which marks the perimeter of infected tissue. Below this is the heavily infected tissue stretching up from the infected root(s).

Transmission electron micrographs taken from the yellow zone (Fig. 54c-d.) show no evidence of hyphae and none of the cells showed any sign of cell wall degradation. However, the cells appeared to be more metabolically active than healthy control cells and the vacuoles were lost in most cases with cytoplasm occupying almost the entire cell. Many organelles could now be identified and there was evidence of considerable activity at the outer membrane indicated by vesicular budding (Fig. 54c-d.). Furthermore, starch grains were absent from most of these cells and where they present, the surface structure appeared altered, suggesting that starch polymers were being mobilised by the host.

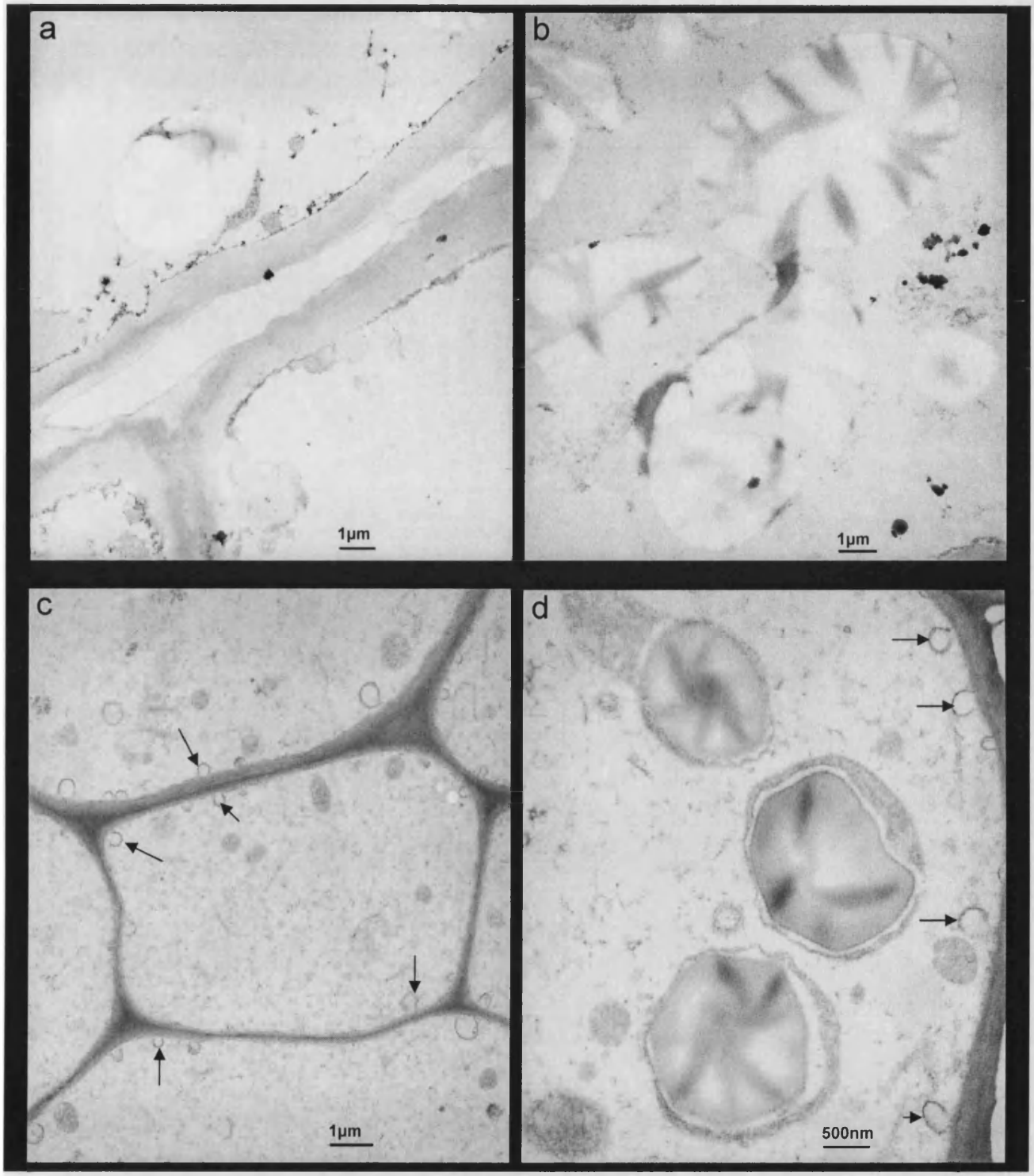

FIG. 54. Transmission electron microscopy of healthy cells and cells immediately in front of fungal ingress from basal tissue of oil palm seedlings. a. Healthy cells from the base of an 18 month old seedling. Image shows the cytoplasm of the cell arrayed around the perimeter of the cell and a large vacuole occupying most of the cell volume. Image also shows a large starch grain; these starch grains are abundant in the basal tissue of oil palm seedlings. **b**. Enhanced magnification of starch grains within healthy cells. **c**. Cells from the 'yellow zone' immediately in front of the 'reaction zone' of an infected oil palm seedling. Cells show no vacuole and cytoplasm occupies most of the cell. There is also extensive vesicular budding (arrows) at the outer membrane and no starch granules are evident. **d**. Starch grains apparently in the process of being immobilised by cellular machinery. Figure also shows vesicular budding from the cytoplasm onto the outer membrane, (arrows).

3.3.8 Infection of Basal Stem Tissue: Reaction Zone

Parenchyma cells of recently infected oil palm basal tissue become filled with *Ganoderma* hyphae. Initially, presumably hyphae utilise cellular components as a food source as there is no indication of cell wall degradation (Fig. 55a). This zone is fully colonised by *Ganoderma* and hyphae take up almost all intracellular spaces but can also grow intercellularly (Fig. 55b). Micrographs of control cells revealed numerous plasmodesmata (Fig. 55c). These occupy localised areas of extremely thin plant cell walls and are perhaps exploited by the fungus for hyphal progression; Fig. 55d shows a hypha in the interaction zone moving between cells at a discrete point whilst the remainder of the cell wall remains remarkably undamaged.

More established areas of infection show tissue similarly packed with *Ganoderma* hyphae but the fungus has perhaps undergone a developmental switch resulting in production of extra-cellular, cell wall degrading enzymes. Breakdown of the host cell wall is shown in close association with a fungal hypha in Fig. 56a-b and breakdown appears to have initiated from a single point where enzymes have been secreted from the fungal hypha. Breakdown is indiscriminate and several cell wall layers have been degraded and is approaching the middle lamella. Degradation of the cell wall occurs and eventually the polymers will be completely destroyed, creating holes through which *Ganoderma* hyphae can pass between cells (Fig. 56c). In many instances cell wall breakdown is not observed in close association with hyphae (Fig. 56d). Secreted enzymes may be discharged and work at distance from the fungus. The figure shows two lesions in the middle lamella of host cells.

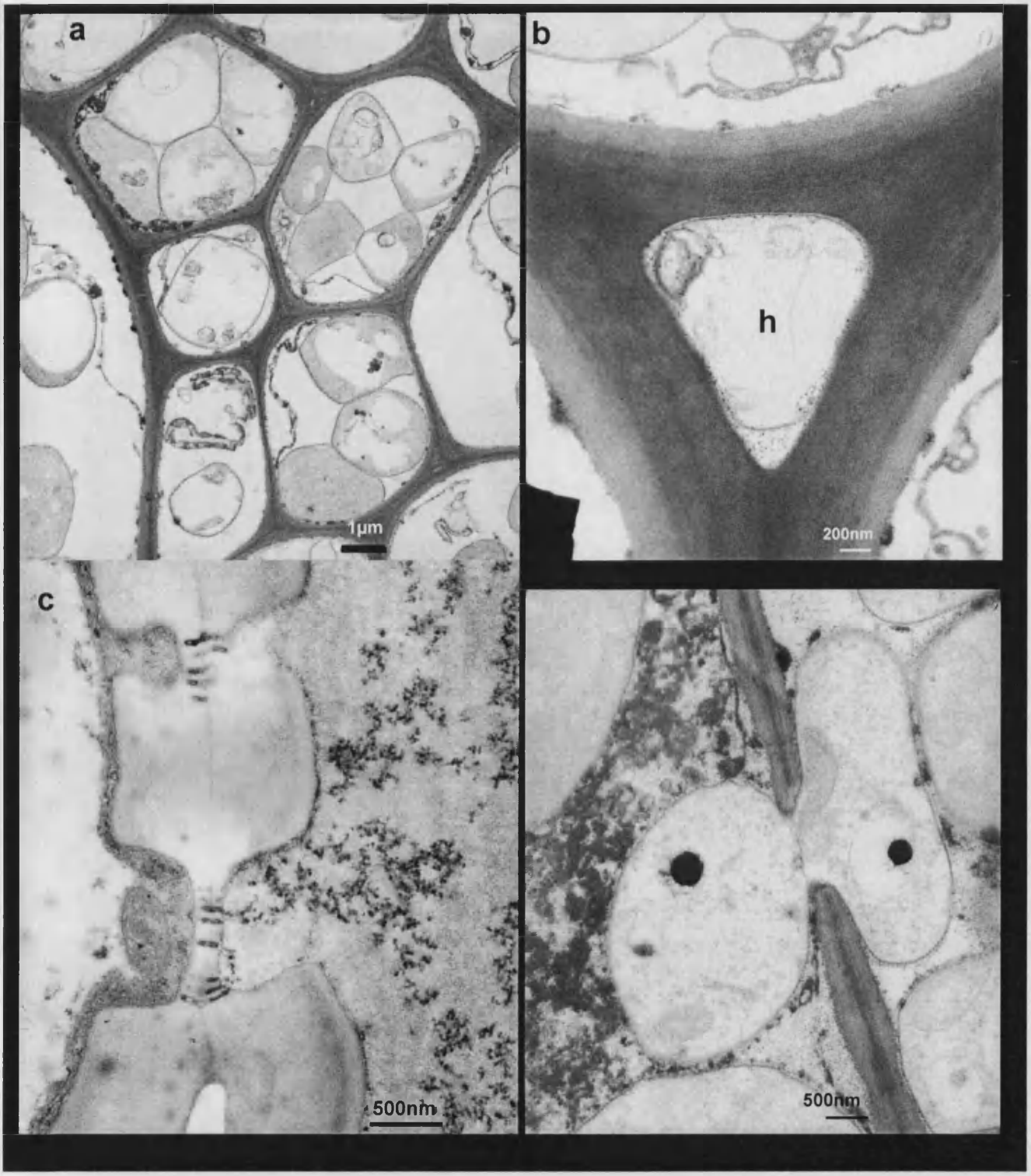

FIG. 55. Transmission electron microscopy from infected and healthy oil palm basal tissue. Transmission electron micrographs of cells from the reaction zone of an 18 month-old oil palm seedling. **a**. Infected oil palm cells packed with thick *G. boninense* hyphae. There are no obvious signs of fungal attack of the cell wall. **b**. *G. boninense* also penetrates intercellular spaces within infected tissue. **c**. Plasmodesmata in oil palm cell wall. **d**. *Ganoderma* hypha advancing between cells through a severed region of the plant cell wall. Localised holes made in the cell wall would perhaps be more easily achieved at regions where plasmodesmata are localised. Hypha=**h**.

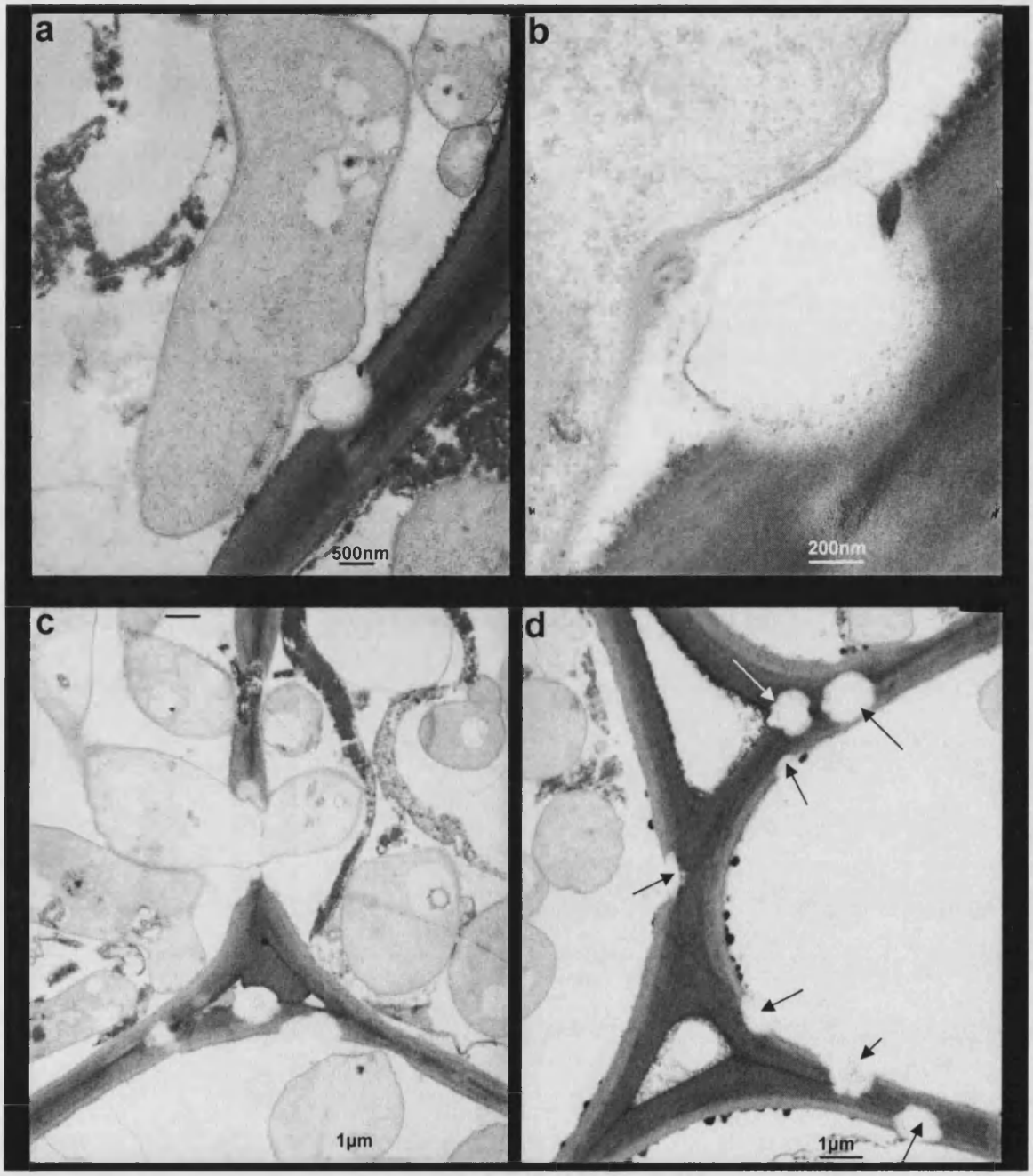

FIG. 56. Transmission electron microscopy of cell wall degradation by *G. boninense* during infection. TEM images of enzymatic attack on plant cell walls of the basal tissue of a 2 yr old oil palm seedling. **a**. Shows hypha in close association to the plant cell wall and a localised area of cell wall degradation. The degradation is localised and includes both primary and secondary cell wall layers. **b**. Enhanced magnification zone of wall degradation in 56a. **c**. *Ganoderma* hyphae packed within host cells in multiple discreet areas of cell wall breakdown where the wall has been completely destroyed and a hypha passing between cells. **d**. Micrograph shows multiple areas of degrading cell walls (arrows). Attack of the cell walls is indiscriminate and involves all wall layers. Many of the areas of cell wall attack are not closely associated with fungal hyphae and may represent breakdown at a distance.

3.4 Discussion

Macromorphological observation of *Ganoderma* development and survival in oil palm tissue confirmed the presence of reaction zones, yellow zones and sclerotia as described by Darmono (51). Reaction zones were first described by Shain (204) as a dynamic interface between living sapwood and decayed wood colonised by the pathogen, however, Pearce (166) and Boddy (30) suggest that these are static boundaries. When reaction zone boundaries fail, a volume of wood is colonised with little or no expression of characteristic reaction zone responses, until ultimately a new reaction zone boundary is established. *G. boninense* decay of oil palm supports this; areas of decayed tissue are delimited by thick-walled melanised hyphae (pseudo-sclerotium) probably in response to formation of static reaction zones. The reaction zone appears to restrict fungal advance and may have elevated levels of antimicrobial compounds. For example, in the reaction zone of naturally infected pruning wounds of the commercial tree species *Eucalyptus nitens*, phenolic gallotannins were upregulated or produced *de novo* compared to healthy sapwood and tannin-protein binding was postulated to play a role in antimicrobial activity of the reaction zone (19). These putative barrier zones are ultimately breached by the pathogen and rapid progress occurs until formation of the next reaction zone, which results in a concentric-like decay pattern, as in USR infections.

The anti-microbial components of the reaction zone may be partially responsible for the formation of pseudo-sclerotia by *G. boninense*, which creates a distinct pigmented margin in BSR and USR infections (51, 98). Sclerotia, or pseudo-sclerotia, are not limited to production by *G. boninense* during pathogenensis; they have also been described from *G. tsugae* growing saprophytically on eastern hemlock (*Tsuga canadensis*) in temperate regions of North America (28) and have been postulated to stop saturation of the wood under high water conditions (27), or conversely they may help prevent desiccation during water stress. Fungal growth has been shown to be dramatically affected at different water potentials (132) and some basidiomycetes have a decreased growth rate at low water content, with a limit of *ca.* 4.4 millipascals (mpa) (29). These melanised structures may also serve to exclude competitive microorganisms from a food source and exclude excess water to maintain conditions favourable for cell wall degradation. They are also formed on the surface of decayed

wood blocks and surrounding infected roots at contact points adjacent to the inoculum source and are termed 'stroma'; these were recently described by Breton *et al* (31) during *G. boninense* infection studies. Ultrastructure reveals thickening of hyphae in these structures and in many cases the cytoplasm has been completely lost. *Ganoderma* can be recovered from these structures from the surface of wood blocks after several months in the soil under greenhouse conditions. However, it is unknown how long *Ganoderma* can remain viable in the soil and if it can survive detached from a food source *in vivo*. If they remain viable for long periods, pseudo-sclerotia may be another potential reservoir of *G. boninense* in the field.

Immediately ahead of the reaction zone in oil palm is an area that was termed the 'yellow zone' by Darmono (51). Darmono suggested the yellow zone was a consequence of fungal activity such as enzyme production, however no evidence of fungal activity or cell wall degradation was observed in this study. The yellow zone probably corresponds to the area Shain described as 'the transition zone' (204) and is likely to involve induced cell wall alterations such as suberisation and lignification which makes cell walls more recalcitrant to fungal CWDEs (138, 164). Ultrastructure of oil palm stem tissue from the yellow zone revealed increased activity of cells in this region and large amounts of vesicular budding onto the outer membrane, which may be associated with alterations to the cell wall. However, together with accumulation of phytoalexins in reaction zones, water potential is also postulated to affect progress of infection by decay fungi (29, 30, 181). Using nuclear magnetic resonance (NMR) Pearce *et al* (167) noted that water levels were elevated in the reaction zone of *Ganoderma adspersum* decay of *Acer pseudoplatanus*. They also noted that the transition zone, adjacent to the reaction zone, had reduced water potential compared with healthy sapwood. They suggested that build-up of elevated water levels in the reaction zone was probably osmotically driven and starch hydrolysis and formation of low molecular weight metabolites around the lesion could be used to generate this. The marked reduction of starch grains in the yellow zone of infected oil palm tissue together with enhanced cytoplasmic activity suggests that this region may play a role in regulating water potential in the reaction zone.

Enzyme production and chemical analysis of degraded oil palm wood *in vitro* reveals that *G. boninense* produces numerous CWDEs. TEM of infected roots and bole tissue

suggests these are likely to be important in degradation of the cell wall during pathogenesis. However, no studies have been conducted to determine which genes are important for pathogenicity. Numerous *Ganoderma* species such as *G. zonatum*, *G. tornatum* and *G. miniatocinctum* have been identified in oil palm plantations (234), however only *G. boninense* causes disease (171) and it is unclear what mechanisms determine pathogenicity. There have been numerous studies to determine pathogenicity factors in fungal infection of herbaceous plants including the rice blast pathogen *Magnaporthe grisea* and the potato blight pathogen *Phytophthora infestans* (221) and ESTs obtained from cDNA of libraries of total fungal RNA during plant infection can be obtained from the COGEME EST database; which now contains 61534 unisequences from 15 phytopathogenic fungi and oomycete species (http://cogeme.ex.ac.uk). Recently Karlsson *et al* (111) produced 923 ESTs from total RNA extracted from mycelium of *H. annosum* during early infection and found that many of the ESTs were common housekeeping genes, but identified some potential pathogenicity factors such as glucanase and superoxide dismutase. Additionally, over 30% of unigenes from *H. annosum* were shown to have no significant homology with known proteins. The authors postulate that these sequences may be unique to basidiomycetes and one of these was a contig of 9 ESTs, which was the most abundantly obtained EST. Similarly, 27.9% of predicted *P. chrysosporium* genes in the published genome sequence had no significant homology to GenBank proteins, supporting the findings of Karlsson *et al* in *H. annosum* and demonstrating the limited current knowledge of basidiomycete genomics (136).

Karlsson *et al* (111) found that a high proportion of ESTs were redundant and this can make it difficult to determine the underlying mechanisms of pathogenicity. For example, loss of cutinase A expressed during infection of gerbera and tomato by *Botrytis cinerea* was shown not to affect pathogenicity (239), whereas disruption of two, closely linked, polygalacturonase genes from the biotrophic fungus *Claviceps purpurea* resulted in almost total loss of pathogenicity (157). Cell signalling proteins are often critical pathogenicity determinants as they influence expression of a number of genes. Mutations in signalling molecules have been used to direct investigation of pathogenicity factors. For instance, where mutations result in loss of pathogenicity, differentially regulated genes in these mutants would be likely virulence factors. In one study, the mitogen-activated protein kinase (MAPK) pathway signalling molecule

MAPK CHK1 was shown to modulate the expression of two cellulase genes; cellobiohydrolase CBH7 and endoglucanase EG6, whose expression was followed by fusing upstream regulatory components with green fluorescent protein to investigate their expression during different phases of infection (GFP) (126). Similarly, mutants of a G-protein (Gα subunit BCG1) involved in signalling in *Botrytis cinerea* showed differential expression of 22 genes including proteases and CWDEs, which may be important in infection (88).

Determination of potential virulence factors in *Ganoderma* could be achieved through production of ESTs from total RNA from mycelium during infection of palm roots, which could then be specifically studied through targeted gene disruption. This would involve development of an efficient transformation system for *G. boninense*. Several transformation methods have so far been described for basidiomycetes including a polyethylene glycol (PEG) mediated transformation of the cultivated mushroom *Pleurotus ostreatus* (127) and restriction enzyme mediated transformation of *Ganoderma lucidum* (114). However, transformation of basidiomycetes mediated by *Agrobacterium tumefaciens* is now assuming prominence and has recently been used to introduce GFP in *H. annosum* with expression shown to be stable over successive subcultures (191). However, this technique has still not been perfected and Burns *et al* (34) reported that introns were required to enhance expression of GFP in *Agaricus bisporus* and *Coprinus cinereus*. Application of this transformation strategy has been carried out in mycoparasitic *Verticillium fungicola* for targeted gene disruption of a β-1,6-glucanase gene *VfGlu1* to assess its affects on parasitism of *A. bisporus* (11). This study showed that although isolates with disrupted *VfGlu1* induced lesions that were significantly smaller than wild type, disruption of this gene was insufficient to stop infection altogether, suggesting that several glucanase enzymes may be encoded by *V. fungicola*. Nevertheless, this serves as a model for potential investigations into pathogenicity factors of *G. boninense* and may be used to investigate putative virulence factors identified from RNA expression during infection as described by Karlsson *et al* (111). An obvious early candidate for the study of virulence factors in *G. boninense* is the enzyme laccase.

During this study biochemical analysis of the oil palm cell walls was used to determine the nature and extent of degradation by *G. boninense* on polymer

components. To accompany this, production of CWDEs by *G. boninense* was analysed from liquid cultures containing oil palm cell walls and from oil palm wood blocks. Polymer analysis of oil palm stems from mature palms revealed that the major components of the stem tissue consisted mainly of cellulose (56%), lignin (18%) and hemicellulose (11%), with pectin (4%) and starch (2%) as minor components. The pectin component of cell walls from non-woody plants can be as high as 35% (140), but in wood, pectin in the middle lamella becomes replaced with lignin and is a minor component (61). However, the low levels of starch are surprising given the extremely high quantity of starch present in mature date palm wood (2).

Biochemical analysis of oil palm wood decayed under laboratory conditions and wood from the base of an infected tree showed rapid and complete removal of starch. There is little information available regarding the influence of starch on pathogenicity, but Coffey (43) reports that the metabolism of starch is altered in plants infected by rust fungi and that initially starch is degraded in a small area of the host surrounding the infection site. Pearce *et al* (167) suggest that starch hydrolysis may be used as part of a host response to generate differential water potential around infection sites. However, it is possible that the ability of *G. boninense* to degrade starch may be important in pathogenicity of oil palm, as it may be an important early food source during *G. boninense* colonisation. Adaskaveg *et al* (2) noted that starch was present in very large quantities in date palm wood, which was commonly degraded by two related *Ganoderma* species: *G. colossum* and *G. zonatum*. Oil palm seedlings also contain a very high starch content (66%) and this might be influential in the development of infection in young palms.

Anomalously, assay of enzyme production showed that there was no α-amylase activity from either liquid cultures or in extracts from wood blocks. The basal tissue of mature oil palm contained only a small starch component, however, it is unknown if starch is found in higher proportions adjacent to the meristem, which may be a factor in USR infections. Since *G. boninense* was able to grow well in liquid cultures with only insoluble starch as a carbon source, production of starch-degrading enzymes would be necessary. It is possible that *G. boninense* breaks down starch using exo-acting enzymes that yield glucose monomers and thus maltose is not normally

catabolised. This may explain the poor growth associated with liquid cultures supplemented with maltose as a carbon source.

As a white rot fungus, *G. boninense* is assumed to produce oxidative lignin-degrading enzymes. Assay of lignin peroxidase (LiP) in fungi is frequently carried out spectrophotometrically by measurement of oxidation of veratryl alcohol to veratryl aldehyde in the presence of H_2O_2, as described by Tien and Kirk (228). Much of the work on degradation of lignin polymers by basidiomycetes has concerned *P. chrysosporium*. However in this study no production of LiP was detected in liquid cultures despite supplementing with veratryl alcohol, tryptophan and syringic acid. Veratryl alcohol has been shown to stimulate lignin-degrading enzymes in studies with *P. chrysosporium* and syringic acid was shown to induce production of laccase in *G. lucidum* (58) whilst tryptophan has been postulated to have protective properties for LiP in liquid cultures (44).

The *P. chrysosporium* genome became the first basidiomycete genome to be completely sequenced (136) and confirmed the presence of ten LiP genes contained within the lignin peroxidase gene cluster (scaffold 85) and five MnP genes, however, no conventional laccases could be identified within the genome. The presence of multiple copies of LiP suggests its importance and it is probable that *G. boninense* also encodes this enzyme. Although activity was not detected from liquid cultures, LiP activity was obtained by extracting enzymes from decaying wood blocks using the Azure B assay developed by Archibald (13), however, assay with veratryl alcohol remained negative. This suggests that LiP enzymes produced by *G. boninense* cannot efficiently catalyse the oxidation of veratryl alcohol to veratryl aldehyde. The fungus was also unable to decolorise poly R-478 dye, an artificial substrate for LiP, on solid medium (data not shown) suggesting that production of LiP by *G. boninense* occurs under specific conditions that are difficult to replicate in artificial systems. d'Souza *et al* (58) found that MnP production by *G. lucidum* occurred only in poplar cultures whereas laccases were produced in both poplar and pine cultures. They suggest that not all classes of lignin modifying enzymes are produced consistently, even in media containing complex lignaceous substrates such as wood.

Gravimetric determination of lignin using the Klason lignin method showed that there was substantial loss of lignin from oil palm wood blocks, which was approximately proportionate to overall dry weight loss. Utilisation of lignin by *G. boninense* is further confirmed by TEM from infected oil palm tissue. Cell wall degradation occurred at discrete locations with attack of all cell layers, including middle lamella regions high in lignin content. Areas of cell wall attack were sometimes not directly adjacent to fungal hyphae; oil palm cell walls in mature tissue are highly lignified and degradation might occur at a distance as the necessarily oxidative nature of lignin breakdown produces reactive oxygen species and phenoxy radicals that may also be damaging to the fungal cell wall (115). Attack of plant cell walls resulting in development of holes through all cell wall layers is indicative of simultaneous wood decay. Similar patterns of cell wall attack have been observed from simultaneous degradation of *Laurelia philipiana* wood by *Ganoderma* sp. (9) and date palm wood by *Ganoderma colossum* and *Phanerochaete chrysosporium* (2). Selective lignin degradation is observed ultrastructurally by the loss of electron density from the middle lamella with less degradation evident from polysaccharide components of the S1 and S2 cell wall layers (9, 28), which is not observed in degradation of oil palm tissue. TEM therefore confirms assumptions that *G. boninense* is a simultaneous degrader of oil palm wood, based on decay of wood blocks *in vitro* and enzymology from liquid cultures and wood blocks.

Despite the difficulties faced in the study of LiP enzymes from liquid cultures, biochemical analysis from degrading wood blocks, enzyme analysis from solid wood blocks and TEM of infected mature oil palm confirms that *G. boninense* produces a battery of lignin-degrading enzymes; it is likely that these enzymes are important in the infection process. Currently there is little information relating LiP activity to pathogenicity and this may be as a result of gene redundancy and difficulties with obtaining expression *in vitro*.

Stimulation of laccase activity was more easily facilitated than LiP and activity was obtained from liquid cultures. Liquid cultures low in nitrogen, with oil palm cell walls used as a carbon source were shown to be most effective at inducing production. However, laccase production was also stimulated in low nitrogen conditions, with a glucose carbon source supplemented with veratryl alcohol. Both laccases and MnPs

have been described in *G. lucidum* but these authors did not describe activity of LiP (58). Activity of LiP and laccases are very similar; both oxidise one electron oxidation, creating radicals. The main difference between laccases and peroxidases are that they utilise different prosthetic groups (LiPs contain heme whilst laccases contain copper as part of their active site) and that laccase generally has a lower oxidation potential than peroxidases (53). Laccases are typically described as lignin modifying enzymes, however, Mayer and Staples (137) have suggested a role for laccases in pathogenicity and reported that laccases may be involved in detoxification of phenolic phytoanticipins and phytoalexins. Cucumber fruits, *Cucumis prophetarium,* contain a family of cyclic triterpenoids, cucurbitacins, and resistance to *B. cinerea* is correlated with the ability of extracts from the fruit to repress laccase secretion (244). Also the phytoalexin, resveratrol, is a phenolic that arises in grapevine in response to attack by *B. cinerea* and a fungal laccase has been shown to detoxify these (8). Additionally, Johansson *et al* determined that aggressiveness of the pine rot pathogen, *H. annosum,* was related to the presence of laccases (109).

Laccase also contributes to pigmentation and production of melanin during infection helps stabilise cell walls and cell turgor pressure and mediates against adverse environmental conditions (104). Formation of melanised, thick cell walled hyphae has been shown to be associated with infection of oil palm in this study. Solid media containing tannic acid has been used to screen for laccase production by microbes (113) and under stress conditions on GSM laccase secretion is evident by oxidation of tannic acid, resulting in discoloration of the agar, from opaque white to brown. Although production of laccase is characteristic of pathogenic isolates, so far production of laccase has not been shown to be required for infection of oil palm. A screen to detect isolates deficient in laccase secretion and subsequent infection trials could help elucidate this. More specifically, targeted gene disruption of laccase in aggressive *G. boninense* isolates could prove a useful tool for determining the role of laccase in pathogenicity.

Analysis of MnP activity was not undertaken during this work and in future, specific assay of this enzyme could be attempted to determine the full range of lignin-degrading enzymes produced by *G. boninense*.

Phytopathogenic fungi produce an array of extracellular cell wall degrading enzymes capable of degrading the complex cell wall polysaccharides of the cell wall and the three main classes of these are: cellulases, hemicellulases and pectinases (46). The most studied of these enzymes in relation to pathogenicity are the pectinases and loss of function can often result in attenuation of virulence. For example, in *Claviceps purpurea*, a biotrophic organ specific pathogen of grasses and cereals disruption of two polygalacturonase genes (*cppg1* and *cppg2*) resulted in almost total loss of pathogenicity (157). Similarly, disruption of a PG in aggressive isolates of *Aspergillus flavus* caused a significant drop in the ability of the fungus to damage and spread within cotton bolls. When this gene was introduced into isolates lacking the gene, the transformants became significantly more aggressive (205). However, the role of pectinases in pathogenicity of woody plants is less well established and virulence of the chestnut blight pathogen, *Cryphonectria parasitica,* did not change with disruption of the enpg-1 gene, which was the major form of PG produced *in vitro* (82). *Ganoderma* also produces pectinases in liquid culture including pectin lyase and polygalacturonase. Pectin is a minor component in mature oil palm cell walls and it is uncertain if these enzymes are critical for infection by *G. boninense*. However, since breakdown of lignin requires a separate energy source, breakdown of limited pectin components may help drive breakdown of lignin.

The role of cellulase and hemicellulases in infection of woody plants is also not fully realised. One of the reasons for the limited information regarding their role in infection could be the presence of numerous isoforms of xylanase, CBH, EG and β-glucosidase. In the non-pathogenic basidiomycete, *P. chrysosporium*, 240 putative carbohydrate enzymes were detected (136). Biochemical analysis of degraded wood and TEM cell wall attack during infection by *G. boninense* confirms the ability of *G. boninense* to attack both the lignin and carbohydrate components of oil palm cell walls. Cellulase production was also detected in liquid medium and assays demonstrate the production of multiple enzymes in the cellulose-degrading complex including EG, CBH and β-glucosidase. Hemicellulose also makes up a sizeable proportion of the oil palm cell wall and xylanases involved in the degradation of hemicellulose polymers are also produced in high quantities during liquid culture.

Chemical analysis of pectin and hemicellulose components of oil palm wood was difficult to ascertain, as the extraction processes were susceptible to contamination by fungal cell wall polymers. The total dry weight of blocks therefore also included a certain proportion of fungal polymers. This was probably comparatively quite small but was not quantified during this project due to time constraints. However, methodology based on quantification of ergosterol content in the wood has been used for determining fungal biomass within decaying wood and other opaque substrates such as soil and leaf material (24, 112, 259) and could be used in future experiments to estimate *Ganoderma* biomass in decaying palm wood. Production of endo-polygalacturonase (PG) and polygalacturonide lyase (PGL) as well as xylanase was detected in liquid cultures of *G. boninense* grown on cell walls.

Numerous *Ganoderma* species have been observed in oil palm plantations (232), however only *G. boninense* appears capable of causing disease of live palms (175). This suggests that *G. boninense* is an aggressive pathogen of oil palm, whereas other species are probably weakly pathogenic or endophytic. Inoculation of palm roots with *G. boninense* results in attachment of fungal hyphae to the roots. Examination of the root surface after one month indicates production of extracellular enzymes by the fungus as revealed by the bleaching and degradation of the tough root outer cell layers. Removal of these tissues allows entry into the cortex of the palm root, which is more readily and systematically degraded. Microscopy shows that the cortex can be almost completely destroyed while the stele remains undamaged, protected by the suberised endodermis. A previous study on root infection by *G. boninense* showed more pronounced infection of vascular tissue, although colonisation of all other tissues was observed (199). In this study, microscopy of infected roots did not concur with preferential utilisation of vascular tissue by *G. boninense*. Progress of the pathogen through root cortical tissues is possibly facilitated by localised degradation of plasmodesmata regions of the plant cell wall. Plasmodesmata cell walls are thinner than other regions of the cell wall and should be easily degraded by CWDEs produced by *G. boninense*.

4 Investigating Biological Control of *Ganoderma* Stem Rot of Oil Palm

4.1 Introduction

Biological control (Biocontrol) can be defined as: the practice or process by which an undesirable organism is controlled by means of another (beneficial) organism (54). In natural environments processes such as competition, predation and parasitism maintain balance within microbial populations. However, human influences can upset these balances and this is most evident when an exotic organism is introduced to new environments (69). Many of the most serious pests and diseases of crops are the result of non-indigenous introductions. Constraining organisms present in the pathogen's region of origin has been termed as "the natural enemy complex" (54). Utilisation of microorganisms to control pests and diseases of plants is accomplished usually by one of the following action: antibiosis, competition, parasitism and induced host resistance (216).

4.1.1 Antibiosis

Weindling first identified antibiotic production by mycoparasitic fungi in 1941 with the characterisation of a "lethal principle" produced by *Trichoderma lignorum*, which he called gliotoxin (248). He demonstrated that this was toxic to *Rhizoctonia solani* and *Sclerotinia americana*. *T. lignorum* was subsequently renamed as *Gliocladium virens* and has been recently renamed again as *Trichoderma virens* (183). *T. virens* is an antagonist of several soil-borne plant pathogens including *R. solani, Sclerotium rolfsii* and *P. ultimum* (216). Isolates of *T. virens* also produce the antibiotic, gliovirin, which is inhibitory to *Pythium ultimum* and *Phytophthora* species. Mutants unable to synthesise gliovirin were unable to control *Pythium* damping off of cotton (105).

The first commercial biocontrol agent (BCA) was the bacterial strain K84 of *Agrobacterium radiobacter,* which has been used successfully to control crown gall disease. Crown gall disease is caused by *Agrobacterium tumefaciens* and infects members of the family Rosaceae such as pear, peach and cherry trees as well as grapevines and is thus a commercial problem. Control of *A. tumefaciens* is brought about by *A. radiobacter* K84, which carries the plasmid pAgK84 encoding the production of a bacteriocin (agrocin 84) and immunity to it. Genetic engineering has been used to remove the transfer region of the plasmid. The genetically engineered strain is called K1026 and is advised to be used in place of K84 to combat the pathogen, as this strain is less likely to transfer immunity to *A. tumefaciens* (169).

Antibiosis is also suspected to play an important role in formation of suppressive soils. Suppressive soils are "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (249). The most well known example of this is the phenomenon of take-all decline (TAD) where development of soils suppressive to *Gauemanomyces graminis* f.sp. *tritici*, the pathogen responsible for take-all of wheat, results in reduction of losses after several years of continuous cropping. *Pseudomonas fluorescens* has been shown to play a role in this phenomenon and it has been shown to produce several antibiotics. These include phenazine-1-carboxylic acid, phenazine-1-carboxamide (PCA), anthracilic acid, 2,4-diacetylphloroglucinol (2,4-DAPG), pyroluteorin, pyrrolnitrin and viscosinomide (229). Strains capable of producing 2,4-DAPG have been found in considerable quantities in TAD soils and it has been suggested that application of these bacteria to areas with high take-all incidence might facilitate more rapid development of suppressive soil (249). The role of *Trichoderma harzianum* isolates in take-all decline has also been studied and the most effective isolate was shown to produce pyrone antibiotics, one of which is also produced by *T. koningii* and *T. hamatum* (216).

However, although antibiotic production is important for many BCAs, it is not always critical. *T. virens* mutants deficient in gliotoxin biosynthesis were just as successful at controlling cotton seedling disease caused by *R. solani* as parent strains. Similarly, mutants deficient for mycoparasitism and gliotoxin synthesis still retained biocontrol

efficacy equal to that of the parent strains (105). These results indicate that while production of antibiotics can be important for biocontrol of plant diseases, it is likely that they are not the only contributing factor and competitive traits leading to greater rhizosphere competence are also likely to be important.

4.1.2 Parasitism

The term parasitism is used broadly to include presumptive fungal mycoparasites (fungi that parasitise other fungi) that coil around other fungal hyphae or overgrow other colonies on agar, and this may involve production of antibiotics, lytic enzymes or toxic radicals (152). The mycoparasite may move towards its target chemotropically and initial attachment is postulated to be through carbohydrates binding to lectins on the target fungus (95). Breaching of the pathogen's cell wall is often presumed to be a necessity for successful biocontrol. Parasitism of the plant pathogen *R. solani* by *T. harzianum* involves coiling of the mycoparasite around hyphae and formation of appressoria (67). Removal of *T. harzianum* hyphae from the surface of *R. solani* reveals holes penetrating through the pathogen's cell wall beneath the appressoria (66). Lytic enzymes such as chitinases, glucanases and proteases have all been shown to be produced by *T. harzianum* and are important factors in its antagonistic properties towards plant pathogens (93). However, lytic enzymes do not work alone and peptaibol antibiotics are postulated to augment their activity by inhibiting host chitin and β-glucan synthesis, such as in the control of *Botrytis cinerea* by *T. harzianum* (131). Although normally investigated for biocontrol purposes, *T. harzianum* is also responsible for green mould infestations of commercial mushrooms, resulting in considerable yield losses of *Agaricus bisporus* (201). Analysis of enzymes secreted by *T. harzianum* biotypes Th2 and Th4 included depolymerases that were shown to attack *A. bisporus* cell walls, trypsin-like proteases, cellulases and isoforms of chitinase that are involved in attacking fungal cell walls (252).

4.1.3 Competition

The ability of BCAs to compete successfully with plant pathogens in the environment is an important component in disease control. Control through competition is

attributed to a number of factors including root colonisation and siderophore production.

The fungus *Idriella bolleyi* has been shown to reduce take-all of wheat by exploiting senescing cortical cells of the wheat plant, where it rapidly produces spores. The spores are then carried down the root by water and continue its colonisation, excluding the pathogen from the root surface (121). Some bacteria have also been shown to colonise the root surface of plants including *Streptomyces griseoviridis* (117) and pseudomonads, which are aggressive root colonisers and result in increased shoot and root growth, leading to use of the term Plant Growth Promoting *Rhizobacteria* (PGPR). A number of factors have been attributed to this, including suppression of major and minor pathogens, fixation of nitrogen and solubilisation of minerals such as phosphorus (216). *Trichoderma* has also been shown to associate closely with cotton roots and is postulated to suppress growth of the pathogen, *Macrophomina phaseolus*. When roots treated with *Trichoderma* were placed in soil colonised with propagules of *M. phaseolus*, only *T. virens* could be recovered from roots on agar. However, when attempted recovery was repeated at 40°C, a temperature at which *T. virens* will not grow, the pathogen grew readily from many parts of the root system (105). Therefore investigations on exclusion of pathogens must be carefully considered and use of fungicide-resistant strains could aid these investigations.

Competition for nutrients in the environment is one of the major growth limiting factors. Ferric iron is especially scarce in the rhizosphere as it has an exceptionally low solubility in water. Both bacteria and fungi produce iron-binding molecules, called siderophores, and these are postulated to be important for biocontrol of *P. ultimum* when assayed *in vitro*. Bacteria applied to seed at the rate 10^7-10^8 cfu/seed and sown in loam naturally colonised with the pathogen were shown to inhibit pathogenicity and had growth-promoting effects. When iron was added in the form of ferric chloride the effect was nullified, implying the role of siderophores in pathogen repression (21). Siderophores are also produced by *Trichoderma* species and these probably play a role in their success as saprophytes in the soil (12).

4.1.4 Induced Host Resistance

Recently much attention has been placed on ability of microorganisms to induce systemic and localised defence responses in plants. The inability of antibiotic production, mycoparasitism and competition to explain completely the biocontrol ability of BCAs has led to the conclusion that other factors may be involved.

The best-studied example of microbial stimulation of elements of the systemic acquired resistance (SAR) pathway is the rhizobacteria-induced systemic resistance (RISR). The RISR pathway resembles the SAR pathway but differs in that it does not result in detectable expression of pathogenesis-related (PR) proteins and often does not lead to accumulation of salicylic acid (95). Induced systemic resistance (ISR) has also been studied in non-pathogenic *Fusarium* species (79) and is postulated to be an important contributor to the build up of *Fusarium* wilt-suppressive soils (249). When plants are pre-inoculated with non-pathogenic isolates of *Fusarium oxysporum*, subsequent inoculation with pathogenic isolates results in mitigation of symptoms. This phenomenon is considered an example of ISR. Most non-pathogenic *F. oxysporum* isolates have been shown to colonise the outer layers of plant roots and this is correlated with the ability to induce resistance in plants. The biochemical responses by plants in response to association with *F. oxysporum* are not clearly understood and studies have implicated elevated production of enzymes related to plant defence reactions, such as chitinase and glucanase, and build up of PR proteins.

Similarly, *Trichoderma* species may induce resistance responses in plants and the first example of this was shown by Birigirama *et al.* where treating soil with *T. harzianum* strain T-39 made leaves of bean plants resistant to diseases caused by *Botrytis cinerea* and *Colletotrichum lindemuthianum* (95). Subsequently *T. harzianum* strain T-203 was tested for its ability to colonise roots of cucumber and its effects on plant growth and pathogen resistance. Microscopy revealed extensive penetration of the fungus into the epidermal cell layers and also to a lesser extent growth within the cortex. Increased production of PR proteins was reported with up-regulation of chitinase and peroxidase enzymes in both the leaves and the roots 48h after inoculation (257). A variety of *Trichoderma* strains, including *T. virens*, *T. asperellum*, *T. atroviride* and *T. harzianum*, have now been shown to induce changes in plants that increase resistance

to a wide variety of plant pathogenic organisms. The response can be non-specific and *Trichoderma* T-22 can induce responses in plants as diverse as tomatoes and maize. Induction of defence responses by *Trichoderma* has been attributed to production of at least three classes of substance that elicit plant defence responses that prevent subsequent infection by pathogens, including peptides, proteins and low molecular weight compounds (95). Although investigation of ISR is at an early stage, it is becoming evident that biological control is a multifaceted process and induction of host resistance may play a greater role than was previously considered.

4.1.5 Biocontrol of *Ganoderma* Basal Stem Rot

Investigation into the use of BCAs to control incidence of BSR in South East Asia has assumed that *Ganoderma* is a soil borne pathogen (217) and mycoparasites have usually been isolated from the soil (1, 197, 217, 218). However, *G. boninese* was shown in this study as a poor competitor outside its preferred environment and has never been isolated free living in the soil. Additionally, use of BCAs as soil amendments for seedlings is similarly unlikely to reduce incidence of disease since palm seedlings are seldom infected in the field except where land preparation has been poor, resulting in large quantities of palm material left in the soil. However, addition of a BCA to the soil may be useful if it can associate with the root system and induce resistance in the oil palm. This is not a trivial requirement however, as the microorganism would have to associate closely with the root surface, grow with the expanding root system and persist in high numbers over a long period of time.

The main source of *G. boninense* inoculum in the field is decaying palm wood and the aim of this study was to isolate potential BCAs from oil palm wood debris with a view to preventing or attacking *Ganoderma* colonisation of this material. Therefore isolation of inhibitory microorganisms and wood decay fungi from rotting palm wood in Indonesia was attempted. Antagonistic microorganisms can be applied to established *Ganoderma* infestations and basidiomycete decay fungi can be inoculated onto fresh tissue to competitively degrade the tissue in order to limit the food source available to *G. boninense*.

4.2 Materials and Methods

4.2.1 Isolate Collection

Mycoparasites were obtained from CABI Bioscience culture collections or isolated in Sumatra using the pre-colonised plate method adapted from (152) (Fig. 57). Petri dishes containing ½ strength PDA were inoculated with *G. boninense* and grown until mycelium had reached the plate margins. Wood samples taken from decaying oil palm stumps with an increment borer or excised from the trunk surface using a sterile knife, were deposited onto the surface of the *Ganoderma* pre-colonised plate. Only antagonistic fungi are able to grow due to lack of attainable nutrients.

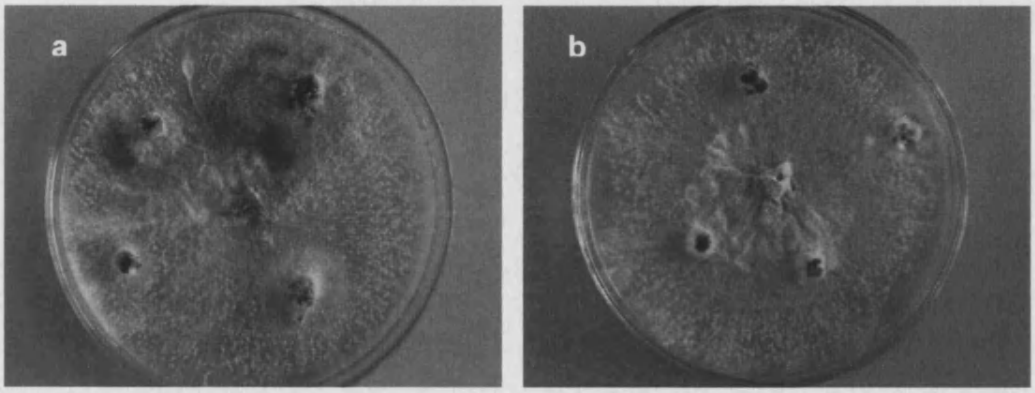

FIG. 57. *G. boninense* pre-colonised plate technique for isolation of mycoparasites. a. ½ strength PDA fully colonised by *Ganoderma* with four cores taken from the trunks of windrows with an increment borer. Green coloured fungi are *Trichoderma* spp. present in the wood cores. **b.** As a, but no obvious macro or microscopic evidence of growth of mycoparasites or antagonists.

Wood degrading fungi were obtained from CABI Bioscience or isolated from degrading palm material, predominantly windrowed oil palm material, in Sumatra. Basidiomycetes were isolated from fruiting bodies. Basidiophores were surface sterilised in 100% ethanol and sections were cut with a sterile scalpel and plated onto ½ strength PDA.

The BCA *Phlebiopsis gigantea* was obtained from the Forestry Commission UK. *Streptomyces griseoviridis* was obtained from the commercially available MYCOSTOP preparation, Verdera, Finland. Culture lists are shown in appendix, Tables 1a-c.

4.2.2 Selective Media

4.2.2.1 *Trichoderma* Selective Medium (TSM)

Selective isolation of *Trichoderma* spp. was facilitated with the medium described by Williams *et al* (253). Basal medium (l^{-1}) consisted of 0.2 g $MgSO_4$. ($7H_2O$), 0.9 g K_2HPO_4, 1.0 g NH_4NO_3, 0.15 g KCl, 0.15 g rose Bengal, 3.0 g glucose, 20.0 g agar mixed in 950 ml distilled water and autoclaved as 121°C for 15 min. Antifungal and fungicidal ingredients were prepared separately and consisted of (l^{-1}), 0.25 g chloramphenicol, 9.0 ml streptomycin stock (1% w/v in SDW), 0.2 g quintozene, and 1.2 ml propamocarb mixed in 39.8 ml SDW. Once basal medium had been autoclaved, the anti-microbial component was added when the temperature had lowered to about 50°C and immediately poured onto petri dishes. All chemicals were obtained from Sigma, except propamocarb, which was acquired from Levington, UK.

4.2.2.2 *Ganoderma* Selective Medium (GSM)

See chapter 2 (15).

4.2.3 Dual Culture

Ganoderma boninense was inoculated onto one half of a ½ strength PDA plate with potential mycoparasites or competitors inoculated at the opposite side and allowed to grow towards each other at 28°C. Once mycelia were almost touching, daily observation of interactions when mycelia came into contact was made. Efficacy of *Ganoderma* killing was assessed by ability to re-isolate *Ganoderma* from areas where mycoparasites had grown over *Ganoderma* mycelium. Re-isolation from equivalent areas in non-mycoparasite colonised plates was used as a control. Absence of *Ganoderma* re-isolation on contact with mycoparasites was interpreted as evidence of destruction of *G. boninense* hyphae. *Ganoderma* was inoculated concurrently with antagonistic fungi or actinomycetes with the exception of the fast growing *Trichoderma* isolates. In this case *Ganoderma* was inoculated 3 days prior to *Trichoderma*. *Ganoderma* and antagonist fungi were inoculated by excising a 1 cm^2 disc from the advancing hyphae of a PDA culture and placing discs mycelium side down on the agar. Inhibition was assessed using methodology adapted from (49), where inhibition was determined by ability to re-isolate *G. boninense* on GSM after contact with antagonists. For fast growing *Trichoderma* isolates re-isolation was

attempted 7 days after initial contact, for slow growing *Gliocladium* and *Clonostachys* isolates re-isolation was attempted 14 days after initial contact.

4.2.4 Preparation of Wood Blocks and Fungal Inoculum for *In Vitro* Inhibition and Comparative Degradation Assays

Oil palm wood or Rubber wood was cut into 3 cm^3 blocks and dried in a drying oven until constant weight was achieved. Blocks were then shaved until each had a dry weight of 6 g ± 0.1 g. Blocks were then rehydrated in SDW for 20 min to *ca.* 70% w/w water content, as found in fresh oil palm wood. Blocks were then sterilised by autoclaving for 1 h at 121°C and placed separately into sterilised 125 ml Nalgene polypropylene containers (Fisher) used as growth chambers. Three millilitres of SDW was added to every inoculated chamber to maintain humidity levels and blocks were raised from the water on glass rods.

4.2.5 Culture of Fungi for Bulk Spore Production

Spore producing fungal antagonists were inoculated on to corn (maize) chips in 1l conical flasks. These were boiled for 10 min to soften and hydrate the grains before autoclaving at 121°C for 20 min. Inoculated corn chips were then incubated in the dark for 1-2 wks to facilitate production of large quantities of conidia. Spores were harvested by mixing inoculated corn chips with SDW. This resulted in a very concentrated spore suspension, which was diluted into a large container to provide 10^6 spores/ml for soil application. Palm seedlings were submersed in the suspension for 2 min/plant to provide standard soil drench.

4.2.6 Preparation of Rubber-Wood Blocks for Field Infection Trials

Large 12x6x6 cm rubber-wood blocks were hydrated by boiling SDW for 30 min and subsequently autoclaved for 1 h at 121°C. Blocks were then inoculated with mycelium from fully colonised *G. boninense* PDA cultures. Typically, large wood blocks become colonised for 9 wks before addition to seedling roots as inoculum. However, for competition inhibition assays using other wood decay fungi,

Ganoderma was only left to colonise the blocks for 6 wks to ensure the food source was not exhausted.

4.2.7 Light Microscopy of Root Sections

Oil palm roots were examined by light microscopy for possible colonisation of roots by *Trichoderma* sp. SBJ8. Roots were cleaned in distilled water to remove extraneous soil and 1 cm^3 root sections were taken with a sterile scalpel. The root was then embedded in SPURR resin and transverse sections made with a microtome were stained with trypan blue (187). Photos were taken with a Nikon Coolpix 995 digital camera.

4.2.8 Degradative Potential of Fungi on Oil Palm Blocks

Growth of degradative fungi on oil palm blocks was carried out using methodology adapted from (7). Palm wood was cut into approximately 3 cm^3 blocks. Blocks were then oven dried at 70°C to constant weight and each was weighed and finally cut to give approximate weight of 5 g. Blocks were then rehydrated in distilled water for 20 min and subsequently autoclaved for 1 h at 121°C. Degradative fungi were inoculated using three 1 cm^2 discs cut from the leading edge of mycelia from potato dextrose agar (PDA). Inoculated wood blocks were placed in sterile Nalgene 125 ml containers in humid conditions at 28°C for periods of 3, 6 and 9 wks after which external mycelium was removed and blocks were oven dried to constant weight to determine weight loss.

4.2.9 Oil Palm Wood Cell Wall Polymer Extraction

Extraction was carried out as previously described, see chapter 2.

4.3 Results

4.3.1 Screening for Antagonistic Fungi *in vitro*

4.3.1.1 Mycoparasites Dual Culture

The simplest way to screen for antagonistic abilities *in vitro* is by using the dual culture method on agar petri dishes. *Ganoderma* was paired against antagonistic fungi and visually monitored for signs of antagonism. Additionally, where *Ganoderma* mycelium was overgrown by the antagonist, re-isolation was attempted using GSM.

Putative antagonistic fungi are listed in Table 3. *Trichoderma* spp were fast growing and in all cases mycelium covered the surface of the petri dish within four days, including areas first colonised by *G. boninense* (Fig. 58). *Clonostachys* and *Gliocladium* spp. were slower growing but still over grew *Ganoderma* mycelium. Although many of the potential antagonistic fungi grew over *Ganoderma* mycelium, in many instances there was no evidence of host destruction. Potential biocontrol fungi isolated from Sumatra, Indonesia, were generally more antagonistic to *G. boninense* than organisms obtained from other sources (Table. 3).

Antagonist Fungi	Re-isolation of *G. boninense* BLRS1	
	Interaction point	Distal edge
Gliocladium catenulatum 288054	-	+
Trichoderma virens 300085	-	+
Trichoderma virens 288567	-	+
Clonostachys rosea APP 0023	+	+
Clonostachys rosea APP 0043	+	+
Clonostachys byssicola AMR 0055	+	+
Clonostachys byssicola AMR 0057	+	+
Trichoderma harzianum APP 0129	-	-
Trichoderma sp. BLRS 3 *	-	-
Trichoderma sp. BLRS 4 *	-	+
Trichoderma sp. BLRS 5 *	-	+
Trichoderma sp. BLRS 6 *	-	-
Clonostachys sp. BLRS 10 *	+	+
Clonostachys sp. BLRS 11 *	+	+
Trichoderma sp. SBJ 8 *	-	-
Trichoderma sp. SBJ 9 *	-	+
Trichoderma sp. SBJ 10 *	-	-
Trichoderma sp. BLRS 13 *	-	-
Trichoderma sp. BLRS 15 *	+	+
Trichoderma sp. BLRS 16 *	-	+
Trichoderma sp. BLRS 20 *	+	+
Phlebiopsis gigantea 1	+	+

Table. 3. Re-isolation of *G. boninense* in contact with and at distances from putative mycoparasitic fungi. Re-isolation of *Ganoderma* was made 7 days after initial contact with *Trichoderma* spp. and 14 days for all other BCAs. * = Fungi isolated from windrows in Sumatra, Indonesia.

Where destruction of *Ganoderma* mycelium was observed, six *Trichoderma* isolates were shown to kill *Ganoderma* both at heavily colonised areas closest to the point of antagonist inoculation and at distal edges: BLRS3, BLRS6, BLRS13, SBJ8, SBJ10 and *Trichoderma harzianum*. These isolates were selected for further assessment of aggressiveness. Of the antagonists obtained from outside Sumatra, only *T. harzianum* appeared to be sufficiently aggressive to merit further study. Two isolates of *Trichoderma virens* and *Gliocladium catenulatum* were also antagonistic to *Ganoderma*, as the host was not recovered from regions first exposed to the antagonist. However, *G. boninense* could still be recovered from distal mycelium, suggesting these fungi are less aggressive against *Ganoderma* than other isolates from Sumatra. The basidiomycete fungus, *P. gigantea,* was also tested for antagonism against *G. boninense* as this organism causes 'hyphal interference' (184) against the temperate basidiomycete *Heterobasidion annosum*, which causes a commercially important disease of pine (root rot) in temperate regions (16, 255). However *P.*

gigantea did not appear to disrupt *Ganoderma* mycelium and a demarcation zone formed at point of contact.

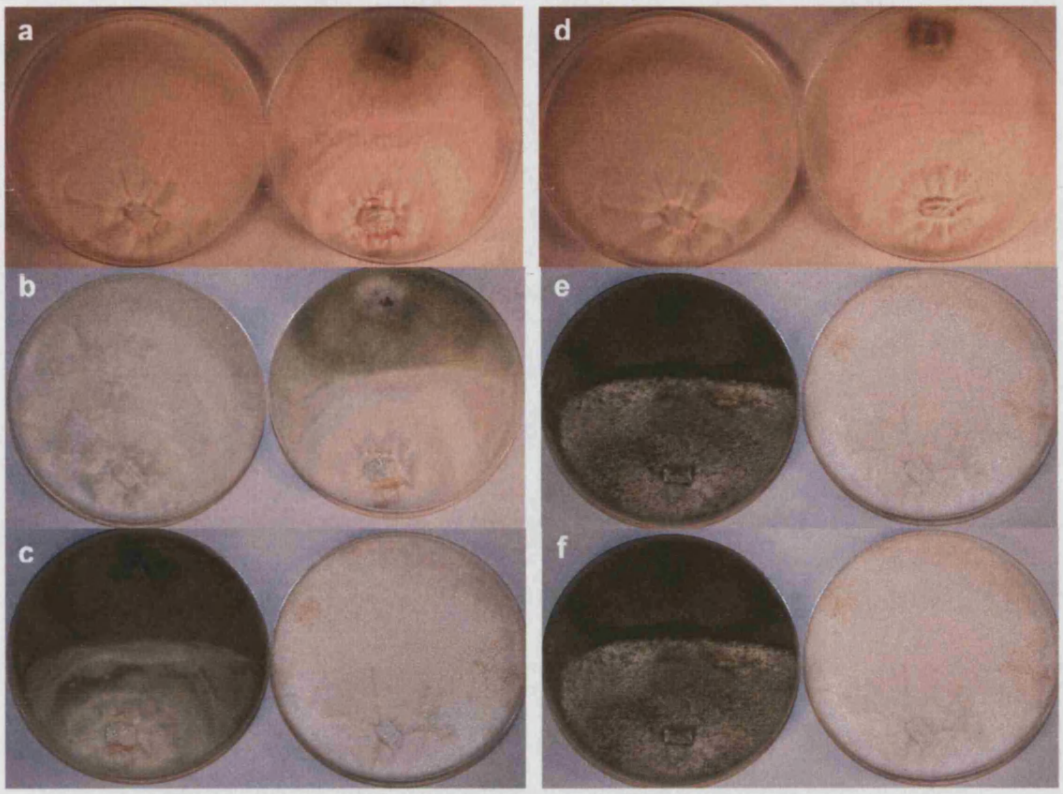

FIG. 58. Dual plates of *G. boninense* vs *Trichoderma* spp. **a**. SBJ1 vs BLRS1, 2 days post *Trichoderma* inoculation. **b**. SBJ8 vs BLRS1, 4 days post *Trichoderma* inoculation. **c** SBJ8 vs BLRS1, 6 days post *Trichoderma* inoculation. **d**. BLRS6 vs BLRS1 2 days post *Trichoderma* inoculation. **e**. BLRS6 vs BLRS1 2 days post *Trichoderma* inoculation. **f**. BLRS6 vs BLRS1 2 days post *Trichoderma* inoculation. Images show *G. boninense* growth is halted as contact with the antagonistic fungi occurs, whilst *Trichoderma* continues to grow and fully envelop the *Ganoderma* mycelium.

4.3.1.2 *Streptomyces* Dual Culture

Actinomycetes are known to produce both antifungal and antibacterial agents (38) and have been effective in control of *Fusarium* wilt of carnation, damping-off of brassicas and root rot diseases of cucumber (220). *Streptomyces* is one of the best studied of these organisms and produces a wide range of anti-microbial compounds. To determine if Actinomycetes produce compounds inhibitory to *Ganoderma*, a commercially available isolate was obtained from Verdara, Finland. The commercial product Mycostop® is provided as 1 g dry sachets and contains approximately10^8 cfu/g *Streptomyces griseoviridis*. The dual plate assay was set up as above on ½ strength PDA, with simultaneous inoculation of host (*Ganoderma*) and antagonist (*Streptomyces*). *Ganoderma* was inoculated as described above and *Streptomyces* was

inoculated by spreading a 10^6 cfu/ml suspension onto the agar using a sterile loop. Three isolates of *G. boninense* were used in the assay: SBJ1, BLRS1 and BLRS2. Plates were observed daily for growth of the organisms and for antagonistic interactions.

After five days *G. boninense* had extended half way across the plate and *Streptomyces* colonies were mature. By 7 days *G. boninense* mycelium from all three isolates had grown most of the way across the petri dish in controls (Fig. 59). However, in the presence of *Streptomyces*, progress of the fungus was halted and inhibition zones formed. After 10 days controls had completely traversed the petri dish. Test plates showed a distinct zone of inhibition, preventing growth of *Ganoderma* within a radius of at least 1cm from *S. griseoviridis* colonies. Soluble anti-fungal compounds were likely responsible for the inhibitory zone. *G. boninense* growth did not resume even after further incubation for two weeks and advanced hyphae became hardened, probably as a result of thickening and melanisation of cell walls. *G. boninense* was readily re-isolated showing that inhibitory compounds did not kill the *Ganoderma* beyond the zone of inhibition.

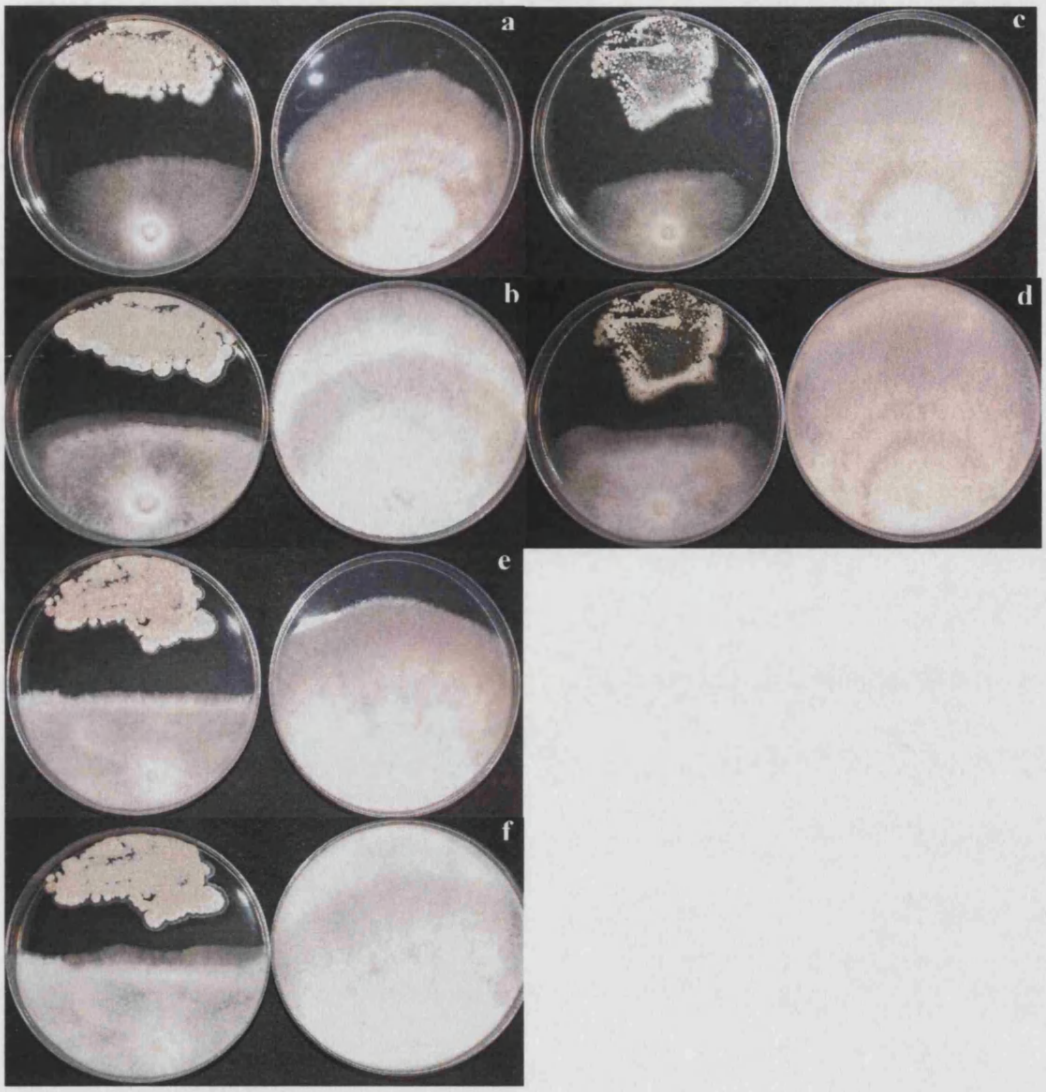

FIG. 59. Dual plates of *G. boninense* isolates vs *Streptomyces griseoviridis*. Both organisms were inoculated concurrently. **a&b**. *G. boninense* SBJ1 vs *Streptomyces*, 7 & 10 days post inoculation respectively. **c&d** *G. boninense* BLRS1 vs *Streptomyces*, 7 & 10 days post inoculation. **e&f**. *G. boninense* BLRS2 vs *Streptomyces*, 7 & 10 days post inoculation.

4.3.2 Inhibition of *Ganoderma* Oil Palm Wood Degradation by *Trichoderma* spp.

Inhibition assays were conducted to determine if mycoparasites identified as aggressive in dual plate experiments were effective at inhibiting *Ganoderma* colonisation of oil palm wood. Four 1 cm^2 PDA discs of *G. boninense* BLRS1 were placed mycelial side down onto oil palm blocks (prepared as above, see 2.2.3) and incubated for 1 wk. Three millilitres of a 10^6/ml *Trichoderma* spore suspension was then applied to the colonising *Ganoderma* mycelium. *Trichoderma* isolates SBJ 8,

BLRS6, BLRS13, BLRS3, SBJ10 and *T. harzianum* were used with 4-fold replication. Four control blocks were inoculated only with *G. boninense* BLRS1 and four blocks were uninoculated. After 6 wks incubation, external mycelial fragments and small tissue samples from the centre of wood blocks were placed onto GSM agar plates to attempt re-isolation of *Ganoderma.* All external mycelium was then removed and blocks were dried to constant weight to determine dry weight loss from blocks. Additionally, to determine if the *Trichoderma* isolates could survive and degrade oil palm wood in absence of *Ganoderma* colonisation, spore suspensions were added directly to wood blocks (4 replicates) and incubated in the dark at 28°C for 6 wks before measuring dry weight loss.

Dry weight loss data from all treatments of *Trichoderma* vs *Ganoderma* blocks was combined and compared against collated weight loss data from blocks treated with *Trichoderma* alone and *Ganoderma* controls. Comparison of means using Student's t-test showed significantly higher degradation of the palm wood by *Trichoderma* alone than in competition blocks (*Trichoderma* vs *Ganoderma*) (Fig. 60). *Ganoderma* control blocks showed the highest degradation of oil palm blocks (32%) compared with 6.8% in *Trichoderma* spp. vs *Ganoderma* blocks and 9% where only *Trichoderma* spp. were applied. In every case where *Ganoderma* mycelium on oil palm blocks was treated with *Trichoderma*, dry weight loss was significantly reduced with comparison to controls (*Ganoderma* inoculation only).

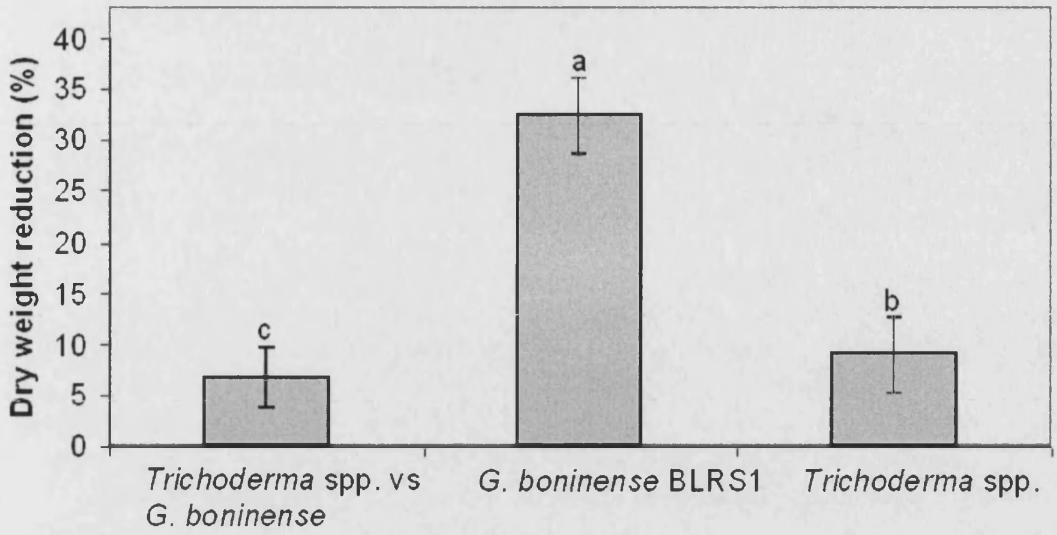

FIG. 60. Degradation of OP blocks (% dry weight loss) after 6 wks by *G. boninense* and *Trichoderma* isolates alone and in combination. The graph shows data as percentage weight loss; this was calculated from gram dry weight loss of each block. Student's t-test was performed on gram weight loss data and letters indicate statistical differences between means (P = 0.05). There were 6 replicates for *G. boninense*. *Trichoderma* replicates were pooled resulting in 24 replicates of *Trichoderma* vs *G. boninense* and 24 replicates of *Trichoderma* alone. Dry weight loss of *Ganoderma* in the absence of antagonistic fungi was 32.44% (sd = 3.82). Dry weight loss was significantly reduced when *G. boninense* was challenged with *Trichoderma* spp. 6.84% (sd = 2.9). *Trichoderma* alone resulted in a dry weight loss of 8.99% (sd = 3.69).

Selection of Isolates for Further Study

Data from several isolates of *Trichoderma* was collated to provide information on the effect of *Trichoderma* addition on *Ganoderma* degradation of oil palm blocks. However, to determine which isolates of *Trichoderma* were most promising for further study, analysis of individual isolates was required. After *G. boninense*, the next highest weight loss was produced as a result of degradation by *Trichoderma* BLRS6 degradation which induced 12% dry weight loss (Table. 4). Generally, blocks treated with *Trichoderma* alone resulted in greater weight loss than blocks inoculated with both *Trichoderma* and *G. boninense*. There was no statistical difference in the ability of *Trichoderma* isolates to reduce weight loss of oil palm blocks by *G. boninense* by comparison of means using Student's t-test. However, *Trichoderma* sp. SBJ8 and *Trichoderma* sp. BLRS13 showed statistically reduced ability to degrade palm wood *in vitro* than other *Trichoderma* isolates.

However, although challenge with *Trichoderma* reduced the ability of *G. boninense* to degrade wood blocks, this did not always correlate with destruction of *G. boninense*.

After 6 wks incubation, only in *Trichoderma* sp. SBJ8 treated blocks was there complete failure to re-isolate *G. boninense* from both external and internal tissue. Also, *G. boninense* could not be re-isolated from external tissue of SBJ10 treated blocks but showed 50% re-isolation from the centre of the wood blocks. All other treatments showed substantial *Ganoderma* re-isolation with BLRS3 the least effective at destroying *G. boninense* mycelium on oil palm wood (Table. 4). Therefore, based on inability to reisolate *G. boninense* and wood degrading potential, 4 isolates of *Trichoderma* were selected for further investigation: SBJ8, SBJ10, BLRS6 and BLRS13.

Block Treatments	(%) Dry weight Loss	(%) External re-isolation	(%) Internal re-isolation
Ganoderma boninense BLRS1 (control)	32.4 (3.8) [a]	100	100
Trichoderma sp. BLRS6	12 (4.4) [b]	NA	NA
Trichoderma sp. SBJ10	11.2 (3.2) [bc]	NA	NA
Trichoderma sp. BLRS3	10.1 (1.2) [bcd]	NA	NA
T. harzianum	9.9 (1.6) [bcd]	NA	NA
Trichoderma sp. SBJ10 vs *G. boninense* BLRS 1	8.7 (3.4) [bcd]	0	50
Trichoderma sp. BLRS3 vs *G. boninense* BLRS 1	8.3 (5.9) [bcd]	75	100
Trichoderma sp. BLRS6 vs *G. boninense* BLRS 1	7.4 (1.7) [bcd]	75	50
Trichoderma sp. SBJ8	5.8 (3.3) [cd]	NA	NA
Trichoderma sp. BLRS13 vs *G. boninense* BLRS1	5.7 (0.7) [cd]	50	75
Trichoderma sp. SBJ8 vs *G. boninense* BLRS 1	5.5 (0.5) [cd]	0	0
T. harzianum vs *G. boninense* BLRS1	5.5 (1.2) [cd]	50	100
Trichoderma sp. BLRS13	5 (2) [d]	NA	NA

Table. 4. Degradation of oil palm blocks (% dry weight loss) after 6 wks by *G. boninense* and *Trichoderma* isolates alone and in combination. Dry weight loss is displayed in percentages; this was calculated from gram dry weight loss of each block. Student's t-test ($P = 0.05$) was performed on gram weight loss data and different letters in the % loss column indicate statistical differences. Table also shows percentage re-isolation of *Ganoderma* from replicates of each treatment. Where treatment involved inoculation of *Trichoderma* alone, no re-isolation of *Ganoderma* was attempted, marked NA = not-applicable. External = re-isolation from mycelium on the surface of the wood block. + = positive re-isolation, - = failed to re-isolate.

4.3.3 Inhibition of *Ganoderma* Oil Palm Wood Degradation by *S. griseoviridis*

The ability of *S. griseoviridis* to inhibit *Ganoderma* degradation of oil palm wood was tested *in vitro*. Oil Palm wood blocks were prepared as above (see 2.2.3) and placed into 125 ml Nalgene containers. A 10^6 cfu/ml suspension of *S. griseoviridis* in SDW was divided and 1 g/l glucose added to half of the suspension. Three blocks were then immediately inoculated with 3 ml *Streptomyces* suspension in glucose and 3 ml *Streptomyces* suspension in SDW (3 replicates per treatment). Blocks were then

incubated in the dark for 1 wk at 28°C before inoculation with four 1 cm^2 PDA discs of BLRS1 placed mycelial side down, onto the wood blocks. Two further treatments comprised: 3 blocks inoculated with BLRS1 + *Streptomyces* and 3 blocks with BLRS1 alone (control). All blocks comprising four treatments were then incubated in the dark at 28°C for 6 wks before measurement of dry weight loss.

Degradation of control oil palm wood blocks was 32%; inoculation with *Streptomyces* suspension supplemented with glucose 1 wk prior to *Ganoderma* significantly reduced the extent of degradation of palm wood by *G. boninense* to only 3.9% (Fig. 61). Inoculation of un-supplemented *Streptomyces* 1 wk before inoculation with *G. boninense* also significantly reduced percentage weight loss of blocks by *G. boninense* (9.2%). Simultaneous inoculation with *Ganoderma* and *Streptomyces* resulted in dry weight loss of 10.7%. Weight loss from blocks after pre-treatment with *Streptomyces* + glucose suspension was significantly less than other treatments.

Re-isolation of *Ganoderma* from control blocks was always possible and consistent re-isolation was achieved from blocks inoculated simultaneously with the host and antagonist. However, *Ganoderma* could only be re-isolated from 50% of blocks treated with *Streptomyces* alone prior to BLRS1 and no successful re-isolations were made from blocks treated with *Streptomyces* supplemented with glucose (Table. 5). This indicates that sufficient build up of *Streptomyces* and probably production of anti-fungal compounds is required for inhibition of *G. boninense* on oil palm wood.

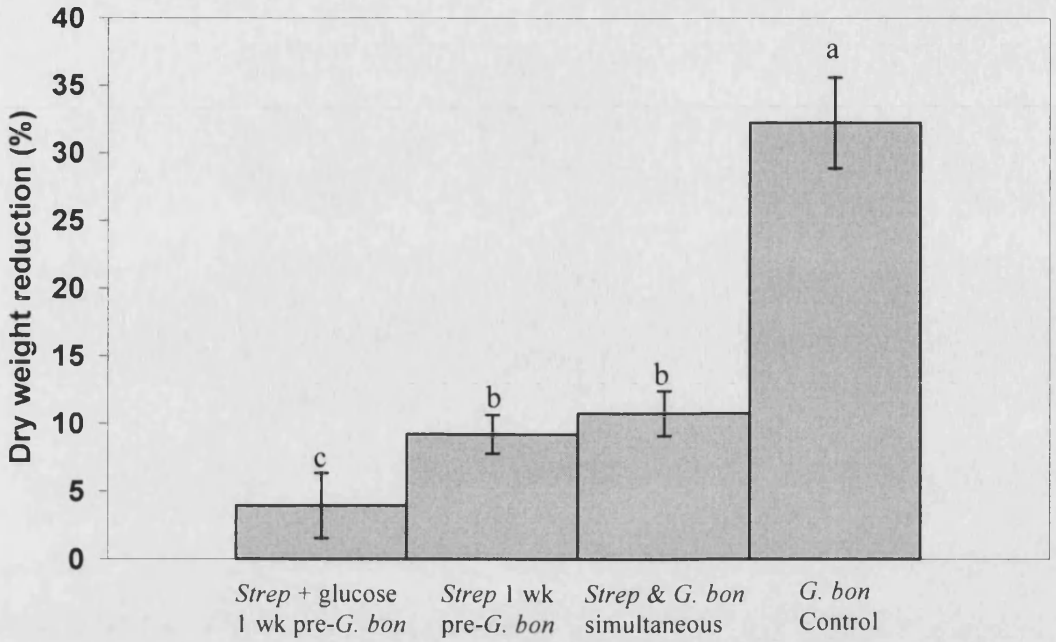

FIG. 61. Inhibition of *Ganoderma* wood degradation caused by *S. griseoviridis*. Dry weight loss is displayed in percentages; this was calculated from gram dry weight loss of each block. Blocks were exposed to the following treatments: control (BLRS1 only), *S. griseoviridis* + glucose inoculated 1 wk before BLRS1 (pre-*G. bon*), *S. griseoviridis* inoculated 1 wk before BLRS1 (pre-*G. bon*), and *S. griseoviridis* inoculated simultaneously with BLRS1. Statistical analysis was conducted on gram weight loss data and showed significant differences by one-way ANOVA ($P < 0.001$, df =3). Significantly different values determined by comparison of means by student's t-test ($P = 0.05$) are indicated by letters above columns. Error bars represent standard deviation of means.

Block Treatments	Internal re-isolation	External re-isolation
S. griseoviridis 1 week pre BLRS 1 (glucose)	-	-
S. griseoviridis 1 week pre BLRS 1 (glucose)	-	-
S. griseoviridis 1 week pre BLRS 1 (glucose)	-	-
S. griseoviridis 1 week pre BLRS 1	-	-
S. griseoviridis 1 week pre BLRS 1	-	+
S. griseoviridis 1 week pre BLRS 1	+	+
S. griseoviridis & BLRS 1 Simultaneously	+	+
S. griseoviridis & BLRS 1 Simultaneously	+	+
S. griseoviridis & BLRS 1 Simultaneously	+	+
G. boninense BLRS1 (Control)	+	+
G. boninense BLRS1 (Control)	+	+
G. boninense BLRS1 (Control)	+	+

Table. 5. Re-isolation of *Ganoderma* from control and *Streptomyces* treated oil palm blocks after 6 wks incubation at 28°C. External = re-isolation from mycelium on the surface of the wood block. + = positive re-isolation, - = failed to re-isolate.

4.3.4 Antagonistic Efficacy of *Trichoderma* spp. against Several Isolates of *Ganoderma* on Oil Palm Wood Blocks *in vitro*

In natural populations, candidate biological control candidates must have broad-spectrum aggressiveness against pathogens. To determine whether candidate biocontrol isolates were effective against multiple isolates of *G. boninense* the *in vitro* block assay was deployed to expose three pathogenic isolates of *G. boninense* to the four most promising *Trichoderma* isolates. *Trichoderma* isolates BLRS6, BLRS13, SBJ8 and SBJ10 were used to inhibit oil palm degradation by *G. boninense* isolates BLRS1, GMR3 and GMB3. Each isolate of *Ganoderma* was used to inoculate 15 oil palm blocks and incubated in the dark for 1 wk at 28°C. The *Ganoderma* inoculation procedure was altered in this experiment to maximise potential for colonisation and prevent rapid desiccation of PDA discs. Blocks were drenched in 3 ml SDW to moisten the block surface immediately prior to inoculation with four 1 cm^2 PDA discs of *Ganoderma* placed mycelial side down onto oil palm blocks (prepared as above, see 2.2.3). Of the 15 *Ganoderma* inoculated blocks, three were not exposed to *Trichoderma* as a positive control. The other blocks were treated with different isolates of *Trichoderma*, three replicates. Blocks were then incubated for a further 5wks before attempting to re-isolate *Ganoderma* from the surface and centre of the blocks and measuring dry weight loss. Growth chambers containing test and control blocks after 6 wks are shown in Fig. 62.

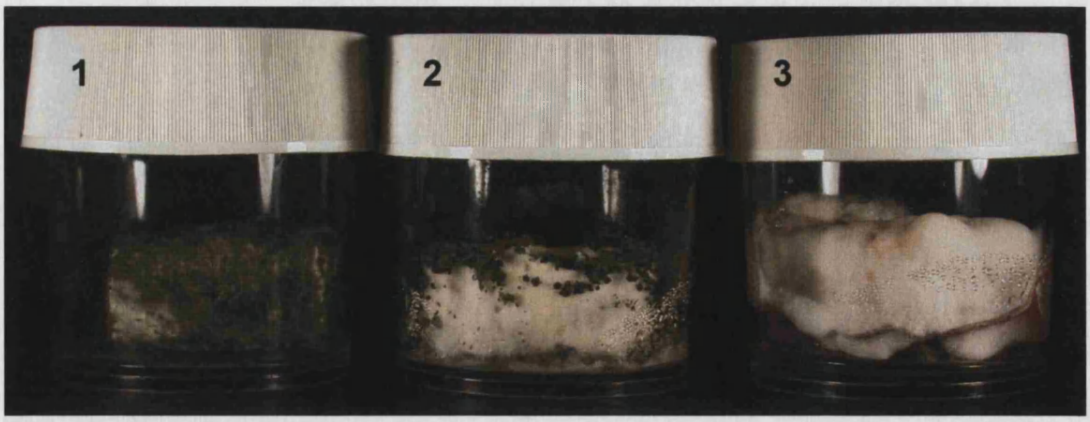

FIG. 62. Comparison of ability of *Trichoderma* isolates to inhibit wood block colonisation by *G. boninense*. Vessel 1 – *Ganoderma* treated with *Trichoderma* sp. SBJ8, vessel 2 – *Ganoderma* treated with *Trichoderma* sp. BLRS13 and vessel 3 – *Ganoderma* control block. Note reduced production of conidia by *Trichoderma* sp. BLRS13 compared to SBJ8.

After 6 weeks, dry weight loss was high from all blocks treated with isolates of *Ganoderma* (37-49%) and there was no significant difference between means of the different *Ganoderma* isolates (Fig. 62). *Trichoderma* isolates SBJ8, SBJ10 and BLRS6 significantly inhibited *G. boninense* wood degradation after 6 wks for each isolate of *Ganoderma*. Inhibition of *Ganoderma* on oil palm was similar for each isolate (17-20% weight loss) with the exception of SBJ 10, which had slightly lower efficacy against BLRS1 (Fig. 63). Re-isolation of *Ganoderma* was not possible from any block treated with SBJ8 or SBJ10 and was only possible from the internal tissue of *G. boninense* BLRS1 when treated with BLRS6.

However, palm wood degradation by *G. boninense* isolates when exposed to *Trichoderma* sp. BLRS13 was not significantly reduced compared with controls (Fig. 63). Furthermore, *Ganoderma* re-isolation was possible in the majority of cases after exposure to this mycoparasite and isolate *G. boninense* GMB3 was successfully re-isolated in every replicate (Table. 6). This suggests that BLRS13 is inhibitory to *G. boninense* but is not as aggressive as *Trichoderma* isolates SBJ8, SBJ10 and BLRS6.

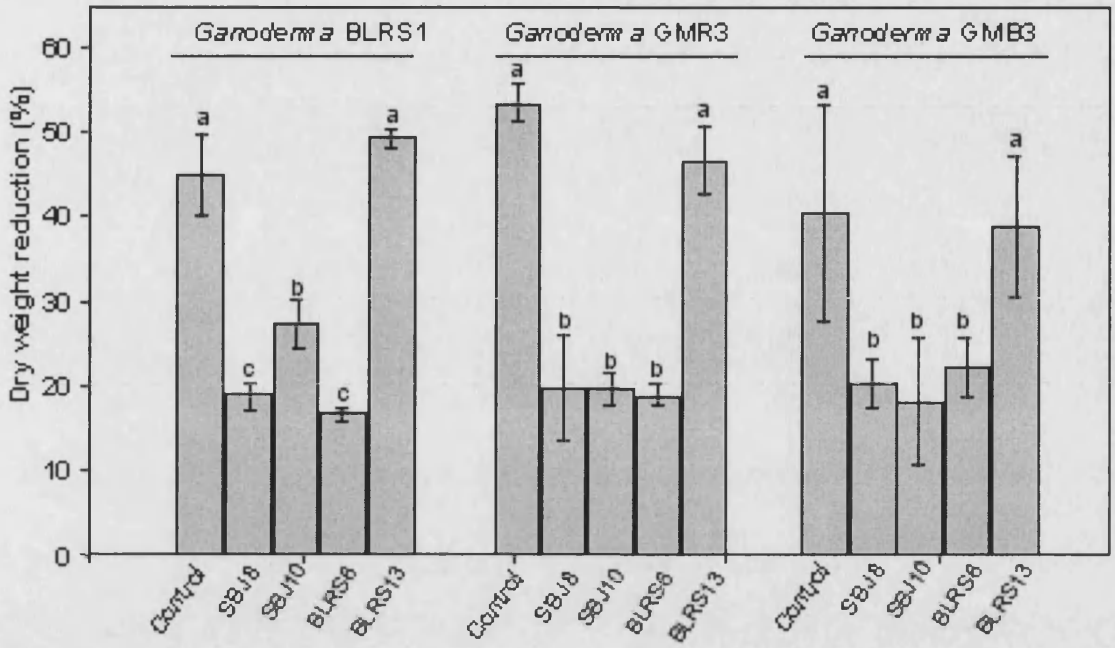

FIG. 63. Inhibition of *Ganoderma* wood degradation caused by *Trichoderma* spp. Figure shows percentage dry weight loss data from blocks after degradation by three isolates of *Ganoderma*, BLRS1, GMR3 and GMB3. Each *Ganoderma* isolate was exposed to the following treatments: control (*Ganoderma* only), inoculation of *Trichoderma* sp. SBJ8, SBJ10, BLRS6 and BLRS13 spores. For each *Ganoderma* isolate tested, statistical analysis of dry weight loss data showed significant difference by one-way ANOVA ($P < 0.0001$, df = 4). Significantly different values determined by comparison of means by student's t-test ($P = 0.05$) are indicated by letters above columns. Mean weight loss of wood blocks by the three different *Ganoderma* isolates was not significantly different ($P = 0.05$). Error bars represent standard deviation of means.

Re-isolation of *Ganoderma boninense*						
	BLRS1		GMB3		GMR3	
Treatment	Internal	External	Internal	External	Internal	External
Control	+	+	+	+	+	+
Control	+	+	+	+	+	+
Control	+	+	+	+	+	+
SBJ8	-	-	-	-	-	-
SBJ8	-	-	-	-	-	-
SBJ8	-	-	-	-	-	-
SBJ10	-	-	-	-	-	-
SBJ10	-	-	-	-	-	-
SBJ10	-	-	-	-	-	-
BLRS6	+	-	-	-	-	-
BLRS6	+	-	-	-	-	-
BLRS6	+	-	-	-	-	-
BLRS13	+	+	+	-	+	+
BLRS13	-	-	+	-	+	+
BLRS13	+	-	-	-	+	+

Table. 6. Re-isolation of *Ganoderma* from blocks after treatment with *Trichoderma* spp. Internal = re-isolation from wood tissue taken from block centre. External = re-isolation from mycelium on the surface of the wood block. + = positive re-isolation, - = failed to re-isolate.

4.3.5 Inhibition of Root Infection by Direct Addition of Antagonistic Microorganisms in Wood Blocks

To determine if challenging *Ganoderma* with potential antagonistic *Trichoderma* isolates can disrupt ability to cause infection, *Ganoderma* colonised rubber wood blocks and colonised blocks challenged with *Trichoderma* were used to inoculate palm seedlings. Rubber wood blocks were used since they were shown to be better inoculum sources than oil palm blocks and were prepared as described previously (see 2.3.5) with exception that growth chambers were changed to 1l Nalgene containers (Fisher), facilitating growth on 15 blocks per container. The most aggressive *G. boninense* isolate, GMR3, was used to inoculate rubber-wood blocks and 4 1-cm^2 discs of mycelium cut from the advancing edge of PDA agar plates was used as inoculum and placed mycelial surface down onto the wood blocks. SDW was added to the interface simultaneously to prevent dehydration of the agar. *G. boninense* was allowed to colonise the blocks for two weeks before addition of *Trichoderma* conidia to the mycoparasite treatment blocks. A 10^6 spores/ml suspension was prepared for *Trichoderma* isolates SBJ8, SBJ10, BLRS6 and BLRS13 and 20 ml added to four Nalgene containers and mixed to ensure each block (15 blocks) was inoculated and 1 container was left untreated (control). Treatment and control blocks were left for a further three weeks to allow *Trichoderma* establishment. Once blocks were fully colonised by *Trichoderma* they were then attached to wounded primary roots using parafilm. Five palms were used for each treatment, with three spatially separated roots inoculated per palm. *G. boninense* infection of oil palm is enhanced by root wounding and this was done to provide optimum conditions for infection in this experiment (2.3.5). Roots were wounded with a sterile scalpel by making a small 1 cm^2 excision to expose the outer cortex. Root infection was checked two months after inoculation and was determined by the presence of advancing necrotic lesions within the root and ability to re-isolate *G. boninense* from diseased tissue. Individual infected roots were scored by assigning binary categories; infected (I) or uninfected (NI).

Seventy two percent of control blocks containing *G. boninense* induced root infection. *Trichoderma* isolates SBJ8 and SBJ10 completely inhibited *G. boninense* infection (Fig. 64), in all 15 blocks. Treatment with *Trichoderma* resulted in infection rates

significantly reduced compared to control and thus the hypothesis that treatment with antagonistic microorganisms has no affect on infection can be rejected (Fig. 64). *Trichoderma* isolate BLRS13 had reduced inhibitory ability in comparison with SBJ8 and SBJ10 and 30% of roots were infected, however this was significantly lower than infection observed from control blocks. *Trichoderma* isolate BLRS6 failed to establish on pre-colonised *Ganoderma* rubber-wood blocks and therefore no data were obtained in this experiment.

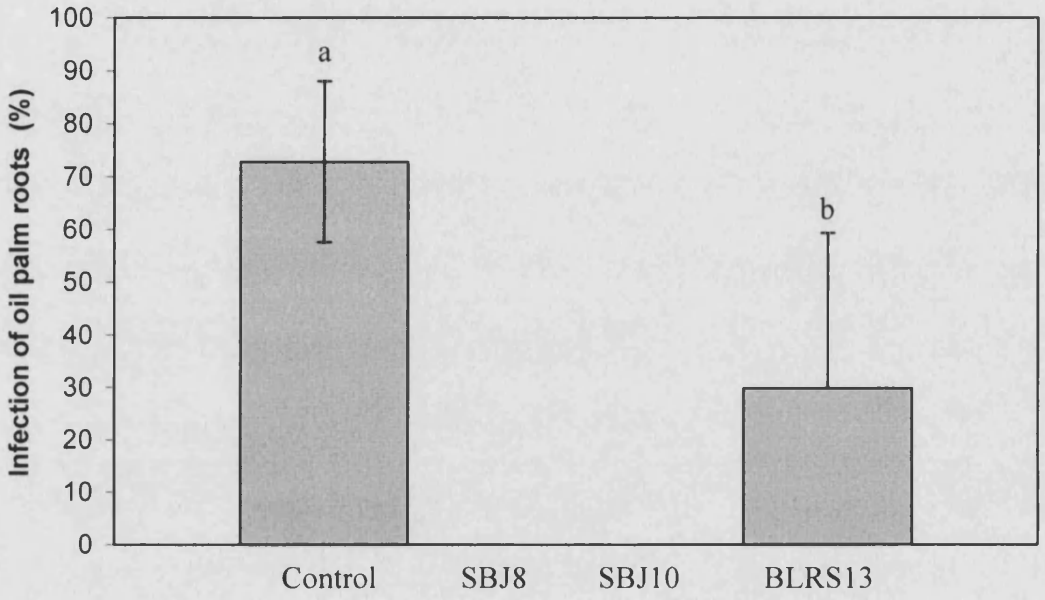

FIG. 64. Infection of oil palm roots after challenge with antagonistic fungi. Number of reps (N), are not identical in every case. On inoculation to roots, only blocks sufficiently colonised by *Ganoderma* were used as inoculum. Of the 15 blocks in each growth chamber, all 15 blocks were completely colonised by *G. boninense* in the control, 14 blocks were completely colonised from the SBJ8 treatment, 12 blocks from the SBJ10 treatment and 14 from the BLRS13 treatment. Error bars represent standard deviation of means. Chi-square analysis (χ^2) analysis for relationship between *G. boninense* infection and *Trichoderma* treatments showed significant differences between control and treatments (P = 0.0001, df =3). As no infection was observed from blocks treated with SBJ8 and SBJ10, these could not be individually compared with controls. Roots inoculated with blocks treated with isolate BLRS13 showed infection in *ca.* 30% of roots; however, this was significantly lower than conrols (P = 0.014, df = 1).

Observation of the rubber-wood blocks two months after attachment to roots (Fig. 65) showed clear differences in the density and nature of *Ganoderma* colonisation between controls and treated blocks. All control blocks showed presence of thick walled, melanised hyphae covering the surface of the wood blocks. Blocks treated with *Trichoderma* SBJ and SBJ10 revealed no *Ganoderma* mycelium remaining on

the wood surface. Treatment with *Trichoderma* BLRS13 shows only 5 blocks with persisting *G. boninense* mycelium. Re-isolation from each block was attempted and is shown in Table 7. All control blocks allowed recovery of *G. boninense*, but no re-isolation was achieved in blocks treated with SBJ8 or SBJ10. Re-isolation of *Ganoderma* was obtained from 5 of 14 BLRS13 treated blocks used to inoculate seedlings.

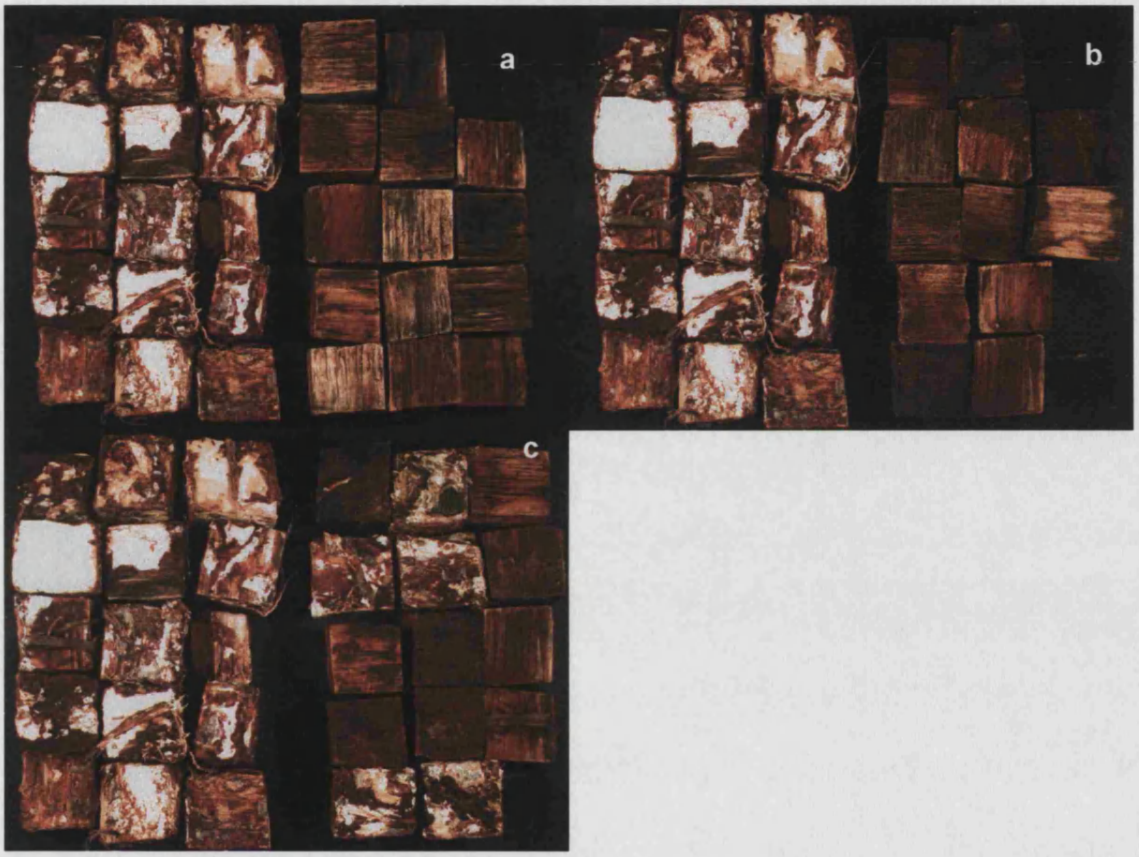

FIG. 65. Control and treated blocks compared 2 months after inoculation to oil palm roots. a. *G. boninense* GMR3 control vs SBJ8. **b.** *G. boninense* GMR3 control vs SBJ10. **c.** *G. boninense* GMR3 control vs BLRS13. Untreated control blocks show *G. boninense* mycelium covering the surface of the blocks in tough, thick cell walled melanised hyphae. Mycelium is almost completely absent in SBJ8 and SBJ10 treated blocks and is present on only 5 blocks treated with BLRS13. Number (N) values are not identical in every case, on inoculation to roots, only blocks sufficiently colonised by *Ganoderma* were used as inoculum. Of the 15 blocks in each growth chamber, all 15 blocks were completely colonised by *G. boninense* in the control, 14 blocks were completely colonised from the SBJ8 treatment, 12 blocks from the SBJ10 treatment and 14 from the BLRS13 treatment.

Treatment	Ganoderma *re-isolation*	
	Positive	Negative
G. boninense (control)	15	0
Trichoderma sp. SBJ8	0	14
Trichoderma sp. SBJ10	0	12
Trichoderma sp. BLRS13	5	9

Table. 7. Re-isolation of *Ganoderma* from control and *Trichoderma* treated blocks 2 months after inoculation of oil palm roots.

4.3.6 Inhibition of Root Infection by Addition of Antagonistic Microorganisms to the Rhizosphere of Oil Palm Seedlings by Soil Drench

This experiment was conducted to determine if application of microorganisms to the rhizosphere of oil palm seedlings two weeks prior to inoculation with the pathogen could reduce incidence of infection. Preparation of blocks and *Ganoderma* inoculum was conducted as above. Once *G. boninense* had been incubated for two wks on rubber-wood blocks, *Trichoderma* isolates SBJ8, SBJ10, BLRS6 and BLRS13 were inoculated onto sterilised corn chips and placed in 500 ml conical flasks for one wk to facilitate bulk production of conidia. Spores were then dislodged into SDW and adjusted to 10^6 spores/ml in a large container. Root balls of palm seedlings were immediately immersed in the suspension for two min. Five palms were used for each isolate of *Trichoderma* and *Streptomyces griseoviridis* and five seedlings were used as untreated controls. Treated palms were left for two weeks to allow establishment of the potential antagonists within the rhizosphere before inoculation of the pathogen to the roots. Roots were not wounded in this case as potential to inhibit *Ganoderma* would necessitate root colonisation, or close association with the root surface. *G. boninense* GMR3 was previously shown to be pathogenic in the absence of root wounding (chapter 2, 2.3.5). Three spatially separated roots were inoculated on each seedling, with parafilm used to attach blocks to the root surface. Root infection was checked two months after inoculation and was determined by the presence of advancing necrotic lesion within the root and ability to re-isolate *G. boninense* from diseased tissue. Individual infected roots were scored by assigning binary categories, infected (I) or uninfected (NI).

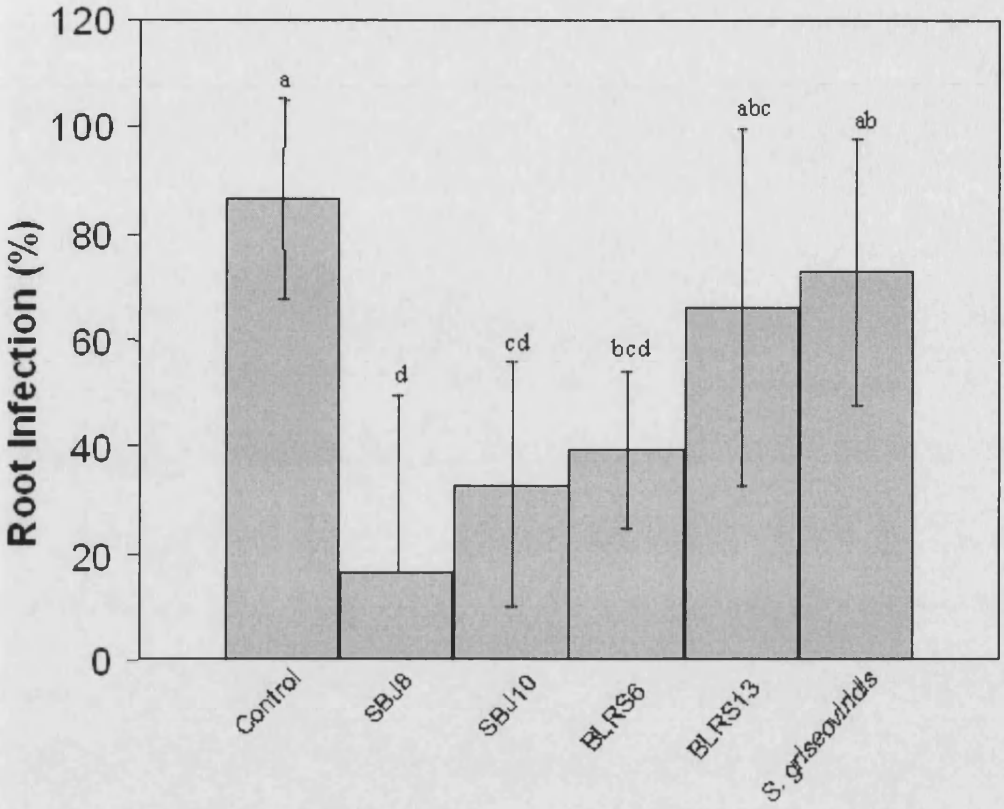

FIG. 66. Effect of pre-treatment of oil palm seedling roots by potential antagonists on infection by *G. boninense*. Analysis of relationships showed significant differences between the treatments (P = 0.001, DF = 5) and the hypothesis, treatment with antagonistic microorganisms has no effect on infection, can be rejected by chi-square (χ^2) analysis. Individual analysis showed that BLRS13 (P = 0.195, df = 1) and *S. griseoviridis* (P = 0.361, df = 1) did not significantly impair root infection compared to controls. Infection levels in the presence of *Trichoderma* isolates SBJ 8 (P = 0.0001, df = 1), SBJ10 (P = 0.003, df =1) and BLRS6 (P = 0.008, df = 1) were significantly lower than controls. *Trichoderma* sp. SBJ8 was significantly the most effective inhibitor of *G. boninense* infection under test conditions. Error bars represent standard deviation of mean root infection from five seedlings (3 infected roots/palm).

Control roots not exposed to soil drenching with antagonists showed 86% infection of roots (Fig. 66). Drenching with *Trichoderma* sp. SBJ8 was most effective at inhibiting *Ganoderma* infection (16% infection), followed by *Trichoderma* sp. SBJ10 (23%) and *Trichoderma* sp. BLRS6 (40%). *S. griseoviridis* and *Trichoderma* sp. BLRS13 were less effective and 72% and 66% of seedlings became infected respectively. There were significant differences between the treatments and thus the hypothesis that drenching soil with antagonistic organisms has no effect on infection can be rejected.

FIG. 67. Control and treatment blocks compared two months after inoculation to non-wounded roots. **a**. *Ganoderma* GMR3 vs *Trichoderma* BLRS6. **b**. *Ganoderma* GMR3 vs *Trichoderma* BLRS13. **c**. *Ganoderma* GMR3 vs *Trichoderma* SBJ8. **d**. *Ganoderma* GMR3 vs *Trichoderma* SBJ10. **e**. *Ganoderma* GMR3 vs *Streptomyces griseoviridis*. Untreated control blocks show *G. boninense* mycelium covering the surface of the blocks in tough, thick cell walled melanised hyphae. In all cases blocks exposed to treatments show traces of *Ganoderma* colonisation but is most prevalent in treatments with *S. griseoviridis* and *Trichoderma* BLRS13. There are only 12 blocks for treatment with *Trichoderma* SBJ 8 as some *G. boninense* blocks were not suitably colonised by the fungus and could not be used in the experiment.

Treatment	% External re-isolation
Control (*G. boninense* GMR3)	15/15
Trichoderma sp. SBJ8	6/15
Trichoderma sp. SBJ10	7/15
Trichoderma sp. BLRS6	6/15
Trichoderma sp. BLRS13	12/15
Streptomyces griseoviridis	12/15

Table. 8. Re-isolation of *G. boninense* from the surface of blocks, 2 months after inoculation to roots.

Visual comparison of *Ganoderma* colonisation of the rubber-wood blocks after two months in the soil showed dense melanised mycelium surrounding control blocks (Fig. 67). Many of the blocks from soil treated with *Streptomyces* and *Trichoderma* BLRS13 also showed dense colonisation by the pathogen. In all treatments, observation of *Ganoderma* colonisation could be seen on a proportion of the blocks. *Ganoderma* was re-isolated from all control blocks and from 12 / 15 of blocks from *Streptomyces* and BLRS13 treatments respectively (Table. 8). SBJ8 and BLRS6 were the most effective inhibitors of *Ganoderma*, as re-isolation was only possible from 6 /15 blocks. SBJ10 was also effective at killing *Ganoderma*, with only 7 / 15 re-isolation.

4.3.7 Persistence of *Trichoderma* Isolate SBJ8 in Soil under Greenhouse Conditions

For application of *Trichoderma* isolate SBJ8 to the soil as a BCA to be effective the fungus must be able to survive for long periods, to associate with the oil palm root surface and to grow with the advancing roots. The first step towards investigating this was to determine how long *Trichoderma* SBJ8 would remain viable in the soil of oil palm seedlings under greenhouse conditions.

Trichoderma isolate SBJ8 was prepared as spores from corn chips. Root balls of five palms were immersed in the spore suspension (10^6 spores/ml) for two minutes each and five palms were left as untreated controls. Sampling was done weekly for the first two months and monthly thereafter. One gram of soil was mixed with 9 ml of SDW and a tenfold dilution made of which 100 µl was added to three TSM agar

plates for each dilution. After two days, colonies were counted with a digital colony counter and converted to cfu/g fresh weight of soil.

No *Trichoderma* was recovered from the rhizosphere of control palms during the course of the experiment and colonies obtained from treated soil were phenotypically indistinguishable from SBJ8. Initially after inoculation *Trichoderma* was present at $3.5x10^5$ cfu/g fresh weight soil (Fig. 68). This increased over the first four wks to a peak of $8.4x10^5$ cfu/g, thereafter colonies in the soil dropped steadily. After 23 wks only $16x10^4$ cfu/g soil was detected and SBJ8 could not be recovered after this time.

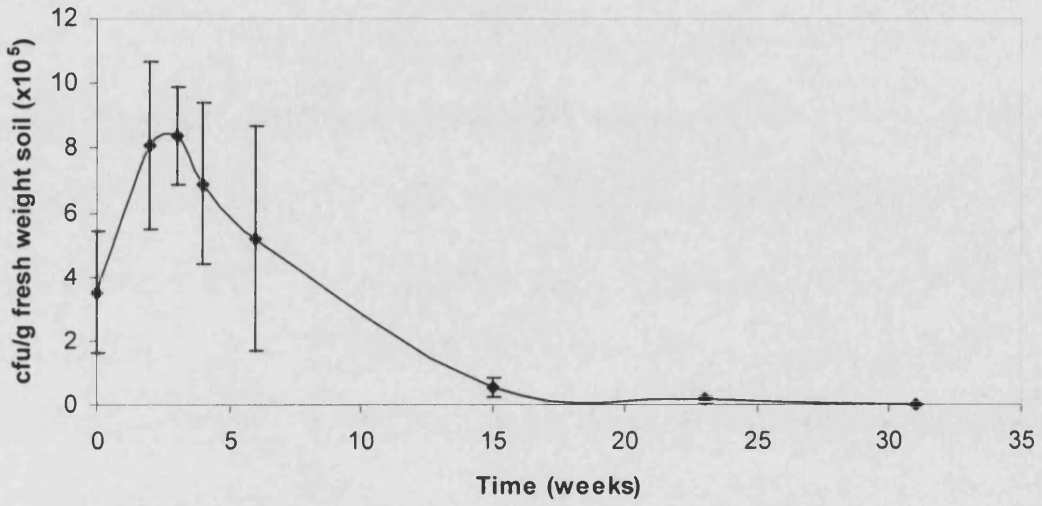

FIG. 68. Persistence of *Trichoderma* SBJ8 in soil/palm rhizosphere under greenhouse conditions.

4.3.8 Oil Palm Root Colonisation by *Trichoderma* Isolate SBJ8

Light microscopy of roots from seedlings one month after exposure to a soil drench of *Trichoderma* sp. SBJ8 revealed fungal structures throughout the root outer cortex, exodermis and epiderm (Fig. 69). There was no evidence of penetration beyond the first few cells of the outer cortex. Fungal structures were also observed on the outside of the root. Since no mycorrhizal fungi were present in the greenhouse conditions, fungal structures were assumed to be *Trichoderma* SBJ8. Control roots not inoculated with *Trichoderma* SBJ8 did not show any evidence of inter- or intracellular fungal association.

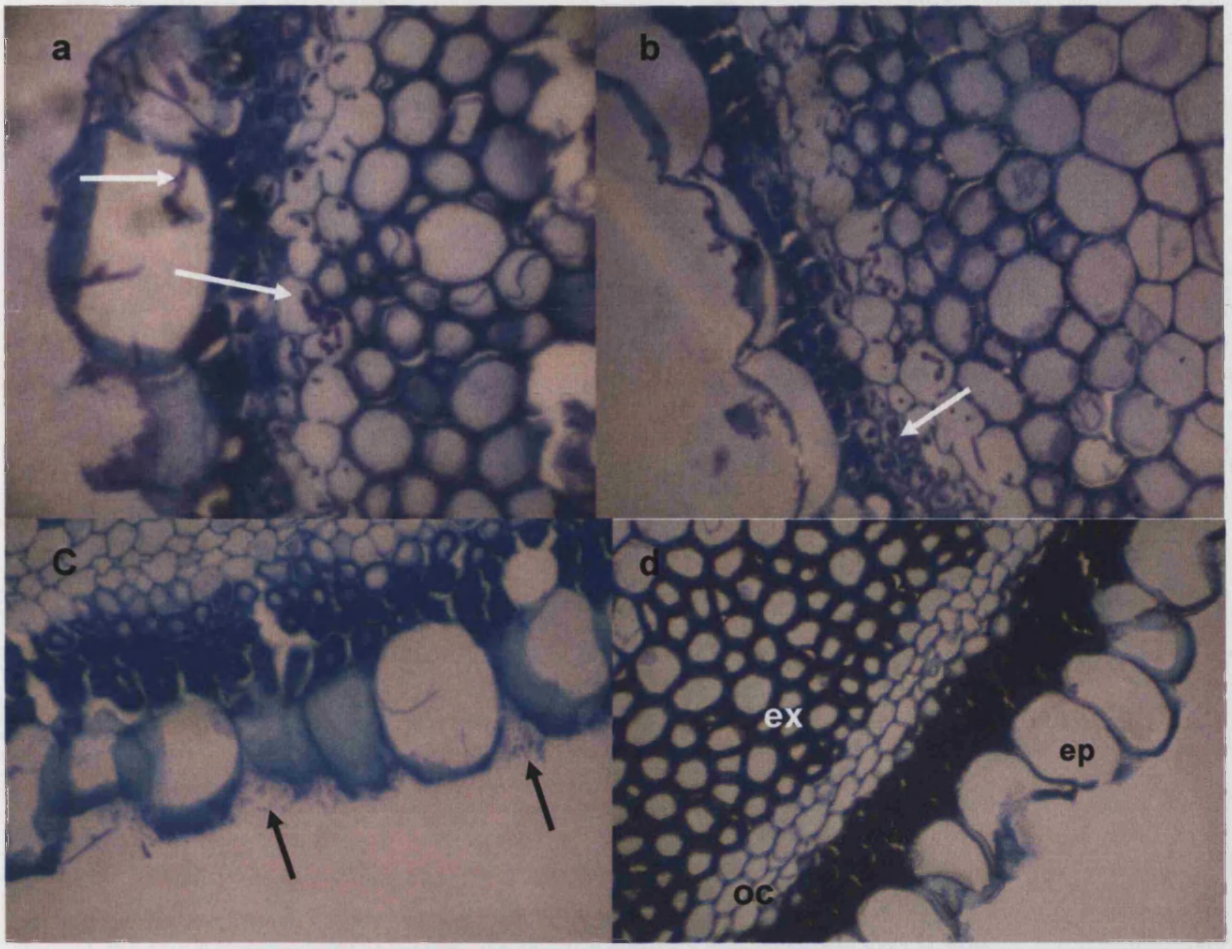

FIG. 69. ***Trichoderma*** **SBJ8 association with outer cell layers of roots from oil palm seedlings.** Light microscopy was conducted using epoxy tissue stain as described (3.2.4.3) (x1000 magnification). **a**. Possible *Trichoderma* colonisation of root outer cortex, exodermis and epidermis. Fungal structures can be seen extensively throughout and are indicated by arrows. **b**. Colonisation of outer cortex. Figure also shows probable colonisation of the exodermis (arrow). **c**. Structures growing on the root surface. **d**. Morphology of uninoculated oil palm roots with no evidence of colonisation of the epidermis (ep), darkly staining exodermis (ex) and outer cortex (oc).

4.3.9 Degradative Potential of Wood Decay Fungi

In order to control *G. boninense* inoculum levels on oil palm debris (trunks, boles) within plantations it is important to obtain competitive wood decay fungi that can be applied to freshly fallen wood debris, colonise rapidly and degrade oil palm wood polymers. In this way pre-emptive application of wood decay fungi could limit the build up of *G. boninense* within plantations through a process of niche exclusion; this has been a successful strategy for many years in the control of root rot of pine (236). Spore suspensions of the weakly parasitic *P. gigantea* are applied to the stumps of

freshly felled pine trees, which colonises stump tissue and excludes *H. annosum* that could otherwise utilise this tissue as an inoculum source for infection of standing pine trees.

Ability of several basidiomycete fungi to degrade oil palm wood effectively was assessed *in vitro* by determining dry weight loss of palm wood blocks induced by fungal growth. Fungi were inoculated onto oil palm wood blocks of known dry weight and incubated in the dark at 28°C for periods of 3, 6 and 9 wks. Three replicates (wood blocks) were used for each isolate per time point. After incubation external mycelium was removed from the surface and dried in an oven at 65°C to constant weight. Weight loss (g dry weight) was converted to percentage dry weight loss and is shown in Table 9.

The polyporous fungus BLRS7, isolated from a windrow at Bah Lias estate, North Sumatra was the most effective degrader after the 9 wk period with 55.7% average dry weight loss from blocks. As expected, *G. boninense* isolates BLRS1 and GMR3 also resulted in substantial weight loss after 9 wks of 54% and 45% respectively. *P. sanguinensis* was comparable to *Ganoderma* isolate GMR3 with 45.4% loss after 9 wks. *P. sanguinensis* was also a rapid coloniser and showed the greatest weight loss (28%) after 3 wks. The only non-native fungus used in the study was *Phlebiopsis gigantea*, a temperate species used as a BCA against pine root rot in Scandinavia and the UK. This fungus also induced high dry weight loss from the oil palm blocks, 42% after 9 wks, despite its lack of adaptation to the substrate. Other fungi that showed promising levels of wood decay were a *Lenzites* sp. with 38% loss after 9 wks, and a *Hydnum* sp., which induced 27% weight loss after 9 wks. Surprisingly, some isolates obtained from decaying oil palm trunks in the field such as a *Marasmius* sp. and *Coprinus* spp. were unable to colonise oil palm blocks under test conditions. Saprophytic growth of these fungi on decaying oil palm material in the plantations of North Sumatra is widespread and they appear to be successful wood degraders under field conditions.

Generally wood degradation appeared to be rapid in the first 6 wks after inoculation. Colonisation of the entire block surface was observed during this time, typically with establishment of a dense mycelial mat covering the external block surface. After 6

wks incubation little further degradation occurred and Fig. 70 shows the profile of degradation by the best three degraders. This is in accordance with a previous study where small oil palm wood blocks were challenged with basidiomycetes and rapid early weight loss was observed (163).

	Percentage Dry Weight Loss		
Strain	**Week 3**	**Week 6**	**Week 9**
BLRS 7	18.26 (4.8)	44.87 (6.0)	55.73 (1.8)
G. boninense BLRS1	17.19 (9.1)	49.55 (3.5)	54.39 (6.5)
Pycnoporus sanguinensis	28.34 (4.7)	43.97 (1.2)	45.4 (2.5)
G. boninense GMR3	23.3 (3.8)	38.7 (9.1)	45.1 (3.3)
Phlebiopsis gigantea	10.78 (12.3)	40.27 (5.0)	41.8 (4.5)
Lenzites sp.	13.43 (2.7)	33.13 (4.5)	38.37 (0.9)
Hydnum sp.	15.39 (2.2)	25.63 (6.1)	26.93 (9.0)
SBJ5	19.52 (2.1)	20.33 (7.9)	22.33 (2.5)
SBJ 4	20.33 (2.7)	20.58 (2.0)	20.75 (0.4)
Trametes hirsuta	7.31 (6.0)	16.8 (6.0)	18.14 (11.1)
SBJ 3	15.39 (1.0)	21.57 (1.3)	18.12 (0.7)
Mycoacia sp.	10.47 (1.8)	18.83 (4.6)	15 (13.7)
Coriolus sp. BLRS20	12.7 (2.6)	9.4 (8.5)	12.71 (2.8)
BLRS12	14.14 (3.8)	14.14 (4.6)	12.98 (2.8)
SBJ2	8.02 (3.0)	8.67 (3.7)	11.5 (1.0)
Corticium sp.	5.13 (1.7)	10.73 (9.5)	8.6 (7.5)
Coprinus sp. BLRS17	NG	NG	NG
Coprinus sp. BLRS9	NG	NG	NG
Marasmius sp.	NG	NG	NG
SBJ6	NG	NG	NG

Table. 9. Degradation of oil palm blocks after 3, 6 and 9 wks by wood-decay fungi. NG = No growth. All values are expressed as percentage dry weight loss. Standard deviation of 3 samples are shown in parentheses.

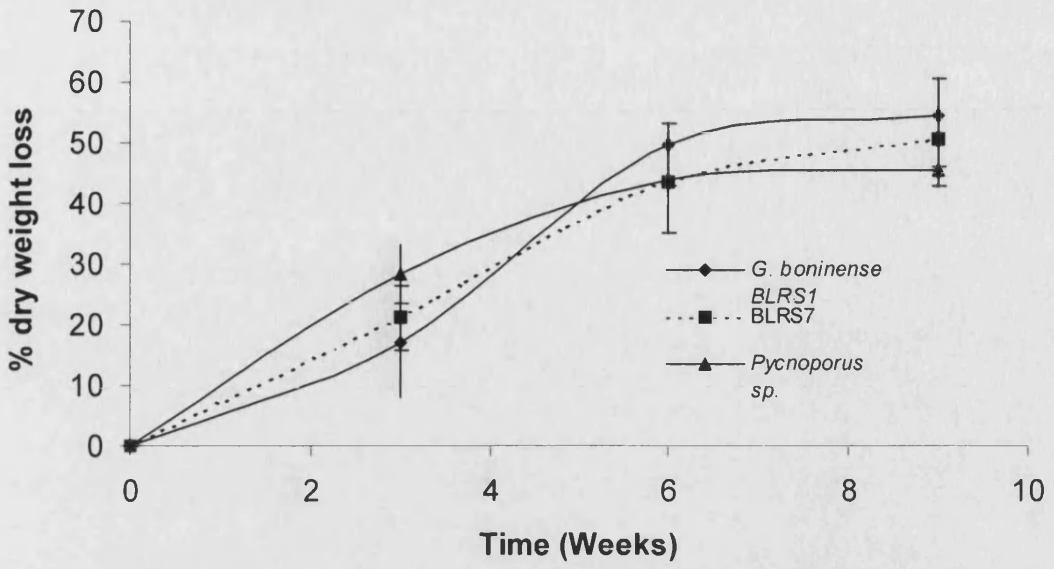

FIG. 70. Kinetics of degradation of oil palm blocks *in vitro* after degradation by *G. boninense*, *P. sanguinensis* and BLRS7 after 3, 6 and 9 wks. Figure shows extensive early weight loss followed by a plateau after 6 wks. Error bars represent standard deviation of mean from three samples.

4.3.10 Comparison of Polymer Utilisation by Selected Wood Degrading Fungi

For use of competitive BCAs to be effective it would be beneficial if potential competitors utilised the same polymer components as *G. boninense* when degrading palm tissue. Two biodegrader fungi, *P. sanguinensis* and BLRS7, were tested to determine if they used wood polymer components in similar proportions as *G. boninense* by chemical analysis of colonised wood blocks after 3, 6 and 9 wks. Polymers were quantified and percentage polymer loss was determined as described (chapter 3) and compared with *G. boninense* isolate BLRS1 over the same time interval. Polymer extraction of degraded wood was complicated by the unavoidable presence of fungal cell wall polymers within the tissue. Treatment with alcohol (alcohol insoluble residue AIR) removed low molecular weight sugars and partial breakdown products. Pectin and hemicellulose extraction processes would have resulted in extraction of fungal polymers, thus contaminating quantification. Starch, cellulose and lignin extraction were unaffected by presence of the fungal polymers.

Mature oil palm wood contains 1.6% starch and after 3 wks incubation starch was completely removed from all blocks challenged with either the degraders or

Ganoderma. Cellulose content in mature oil palm tissue was 56%. *Pycnoporus* degraded cellulose less than the other fungi, with a 35% percent weight loss after 9 wks (Fig. 71). *G. boninense* and BLRS7 induced 57% and 59% cellulose weight loss after 9 wks. The lignin component of mature oil palm wood is 18% and this was reduced *ca.* 45% within the first 3 wks by *P. sanguinensis* and by 60% over the 9 wk period (Fig. 72). Rate of lignin loss from blocks inoculated with *Ganoderma* and BLRS7 was lower in the first 3 wks at 27% and 12% respectively. However, this was to be expected since dry weight loss was highest in blocks degraded by *Pycnoporus* over this time interval. After 9 wks, percentage lignin loss for *G. boninense* and BLRS7 was 55% and 59% respectively, which was comparable to *P. sanguinensis*. Overall it is apparent that the fungi all attack both lignin and holocellulose content in wood simultaneously.

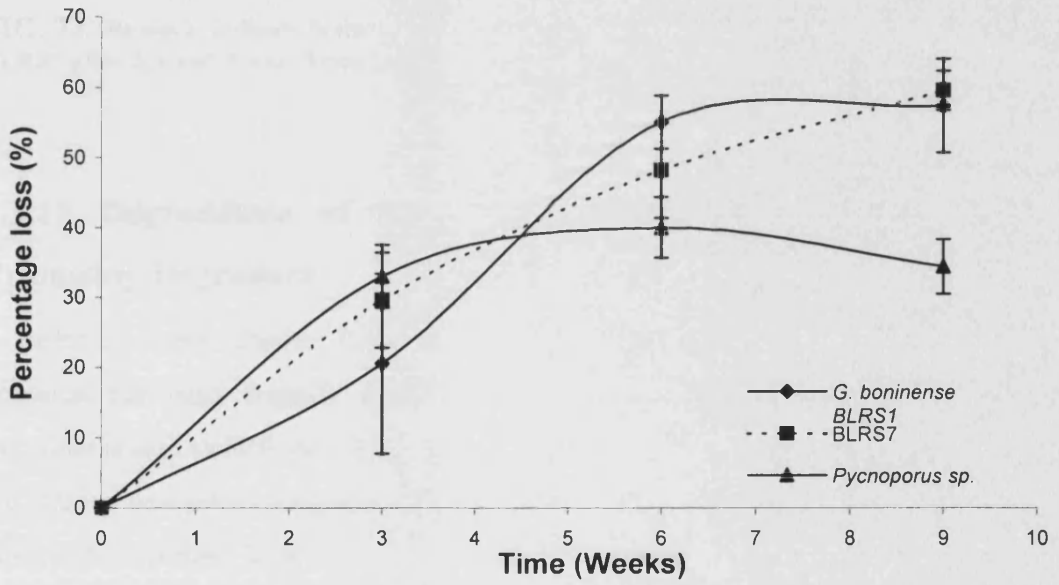

FIG. 71. Removal of cellulose from oil palm blocks *in vitro* by *G. boninense*, *P. sanguinensis* and BLRS7 after 3, 6 and 9 wks. Error bars represent standard deviation of mean from three reps.

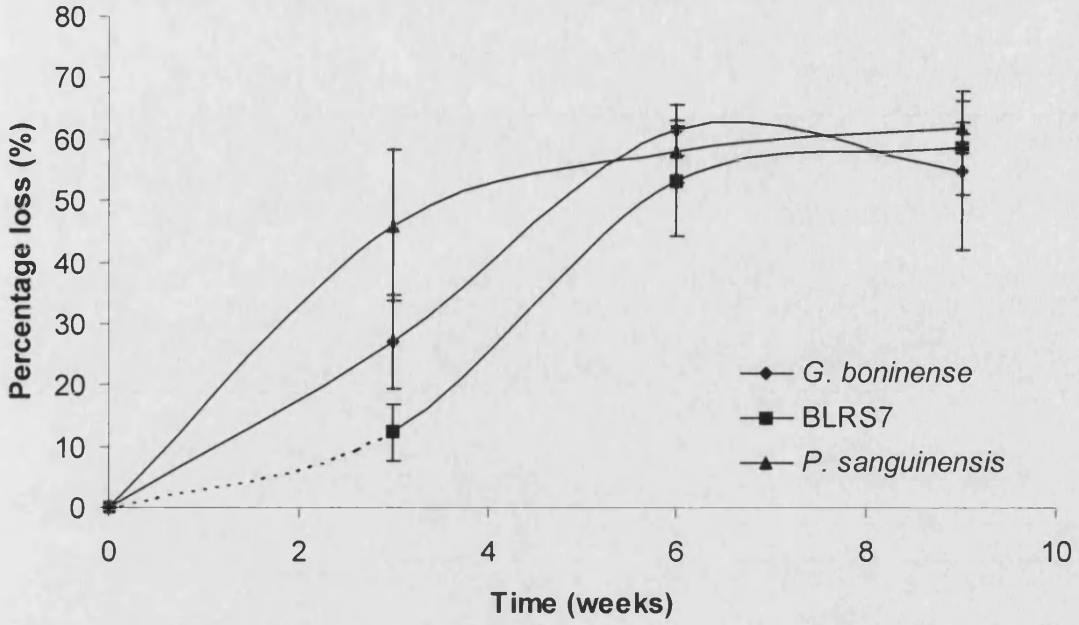

FIG. 72. Removal of lignin from oil palm blocks *in vitro* by *G. boninense*, *P. sanguinensis* and BLRS7 after 3, 6 and 9 wks. Error bars represent standard deviation of mean from three reps.

4.3.11 Degradation of Palm Wood Under Field Conditions by Promising Degraders

In order to assess whether Basidiomycete fungi screened in the lab for degradative potential can also degrade palm wood significantly in the field, the following experiment was carried out. Five isolates of biodegradative fungi were to be tested in the field: *Pycnoporus sanguinensis*, *Trametes hirsuta*, *Lenzites* sp., *Hydnum* sp. and unidentified isolate BLRS 7. Newly felled palm wood showing no symptoms of *Ganoderma* infestation was cut into large discs, approximately 15 cm in width. The bole and base of the trunk up to 0.5 metres were excluded from the experiment. Wood adjacent to the meristem from the topmost part of the palm was also excluded from the experiment because of texture and compositional differences compared with basal wood. Trunk sections were weighed, placed in 1.5 x 1 m nylon mesh sacks and immediately inoculated with the degradative fungi. Fungi were grown in bags containing 300 g of corn chips for a period of two weeks before applying to the cut palm wood. Sixteen discs were inoculated with each fungus and 16 discs were left uninoculated as controls. To each section, one bag of colonised corn chips containing

300 g of fully colonised corn chips was used as inoculum. Discs were stacked in piles (Fig. 73) of four in shaded conditions; this increased contact between the cut surface and inoculum, and shaded conditions minimise extremes of temperature. An estimate of the dry weight of each block was obtained by accurately weighing two wood samples (approximately 200 g) from 5 uninoculated discs. Samples were then weighed (fresh weight) and placed in a drying oven until they reached constant weight. The final dry weight and initial fresh weight of the samples was used to determine average water content for each block and the mean water content for the discs was used to estimate the dry weight of each disc used in the experiment. All discs were then weighed again after 6 and 20 wks and an accurately measured sample (200 g) cut from each disc and dried to constant weight. Sample dry weight values were used to determine dry weight loss of wood discs. After determining dry weights of the samples, they were replaced in the mesh sacks containing the decaying discs.

FIG. 73. Stacked discs from degradation *in situ* experiment. Picture was taken during set-up of the experiment. Discs weigh approximately 20 kg (fresh weight) and are placed into a mesh sack to allow collection of wood fragments for weight measurement when assessing degradation. There are 8 discs for each degrader isolate and were then transported to two shaded locations, 4 discs from each treatment per location.

Dry weight loss from the discs occurred rapidly after fungal inoculation and after 6 wks substantial degradation had already occurred (Table. 10). Throughout the

experiment, weight loss was greatest in blocks treated with *Trametes hirsuta*, which induced dry weight loss of 29.8% after 6 wks rising to 39.8% after 20 wks. *P. sanguinensis*-inoculated blocks also showed similar weight loss, 28.1% after 6 wks rising to 40.1% after 20 wks. *Hydnum* and *Lenzites* sp., inoculated blocks showed a dry weight loss of *ca.* 34% after 20 wks. Uninoculated control blocks showed the lowest dry weight loss after 6 and 20 wks, but after 6 wks dry weight loss in discs inoculated with BLRS7 and *Hydnum* sp. showed less dry weight loss than controls. However, there was no statistically significant difference between the treatments after 6 or 20 wks (Table 10).

Fungus	Percentage Dry Weight Loss	
	Week 6	Week 20
Trametes hirsuta	29.8 (18.3)	39.8 (19.4)
Pycnoporus sanguinensis	28.1 (16)	40.1 (26.2)
Hydnum sp.	25.5 (17.2)	34.3 (20.9)
Lenzites sp.	28.1 (15.7)	33.6 (15.4)
BLRS7 (polypore)	23.7 (10.6)	26.7 (13.6)
Uninoculated (control)	27.7 (7.9)	26.3 (15.8)

Table. 10. Percentage dry weight loss of oil palm discs inoculated with different wood-decay fungi after 6 and 20 weeks. Standard deviation shown in parentheses. Statistical analysis of dry weight loss using one-way ANOVA showed no statistical differences between treatments after 6 ($P < 0.36$, df = 5) and 20 wks ($P < 0.314$, df = 5). Comparison of means by Tukey-Kramer HSD ($P = 0.05$) also showed no statistical differences.

4.3.12 Inhibition of Root Infection by *G. boninense* due to Competitive Biodegrading Fungi

A field trial was set up in Sumatra to determine if competition from wood decay fungi in inoculum source blocks can inhibit infection. Large, 12x6x6 cm rubber wood blocks were prepared as described and inoculated with *Ganoderma*. Incubation time was reduced from 9 wks to 6 wks to ensure that the food source had not been completely utilised by *Ganoderma* when challenged with degradative fungi. Blocks were added to soil after 6 wks and surrounded by corn chips colonised with different biodegradative fungi: *P. sanguinensis*, unidentified polypore BLRS7, *Trametes hirsuta*, *Lenzites* sp. and *Hydnum* sp. Three-month old oil palm seedlings were then

placed above the inoculum and soil added to the seedling bag to complete the planting. Ten replicate seedlings were inoculated for each wood decay fungus and seedlings were placed in shaded conditions to promote favourable conditions for *Ganoderma* infection (Chapter 1). Seedlings were then observed monthly for signs of infection based on foliar symptoms and data recorded; percentage infection of seedlings is displayed in Fig. 74. For analysis infection was recorded as, I = infected or N = not infected.

Seedlings planted in soil containing uninoculated control blocks showed no infection in any of the palms after 6 months. Blocks inoculated with *Ganoderma* resulted in infection of *ca.* 60% of seedlings after 6 months. Greatest infection rates (*ca.* 70%) were observed in seedlings exposed to the treatments *Ganoderma* + *Pycnoporus*, and *Ganoderma* + *Lenzites* sp. The lowest infection (30%) occurred in seedlings treated with *Ganoderma* and *Hydnum*. However, there was no significant difference between infection frequency of *Ganoderma* controls and treatments (Fig. 74).

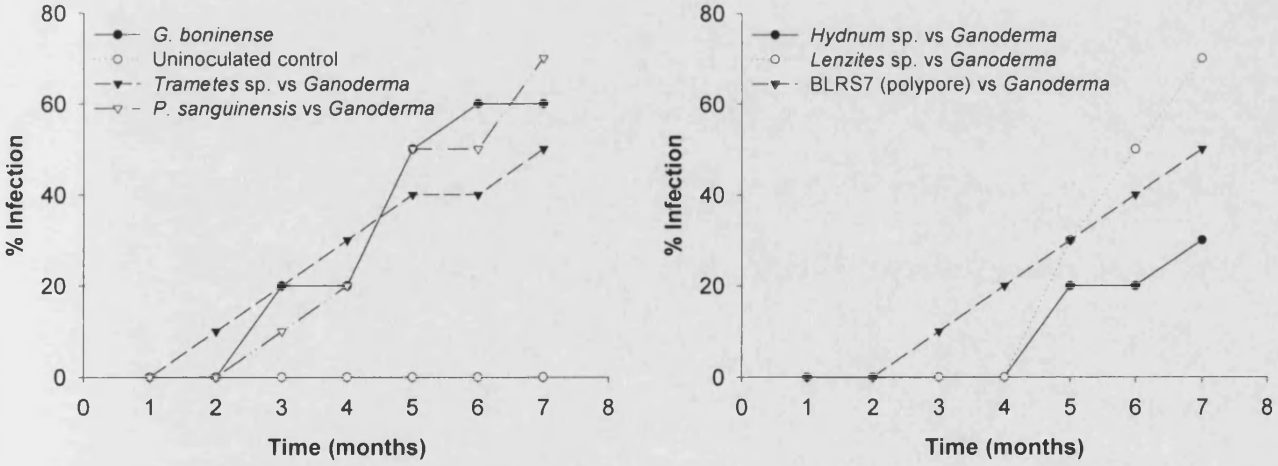

FIG. 74. Infection of oil palm seedlings by *Ganoderma* in the presence and absence of wood-degrading basidiomycetes. No significant differences were observed between *Ganoderma* controls and treatments ($P < 0.461$, df = 5) determined by χ^2 analysis.

4.3.13 Determining Potential Pathogenicity of Degradative Fungi

None of the fungal isolates considered as potential competitive BCAs are known pathogens of oil palm. However under stress conditions or artificially high inoculum

levels the potential of any possible biocontrol isolates to cause disease should be assessed.

In Sumatra uninoculated 12x6x6 cm rubber-wood blocks were placed in soil and surrounded by corn chips colonised with different degradative fungi: *P. sanguinensis*, unidentified polypore BLRS7, *Trametes hirsuta*, *Lenzites* sp. and *Hydnum* sp. Ten replicates were made for each isolate and compared with a positive control comprising *G. boninense* colonised corn chips used as an inoculum for the blocks and a negative control consisted of uninoculated rubber blocks placed in the soil (ten replicates). Three-month old oil palm seedlings were then placed above the inoculum and soil added to the seedling bag to complete the planting. Seedlings were then observed monthly for signs of infection based on foliar symptoms and data collected as above.

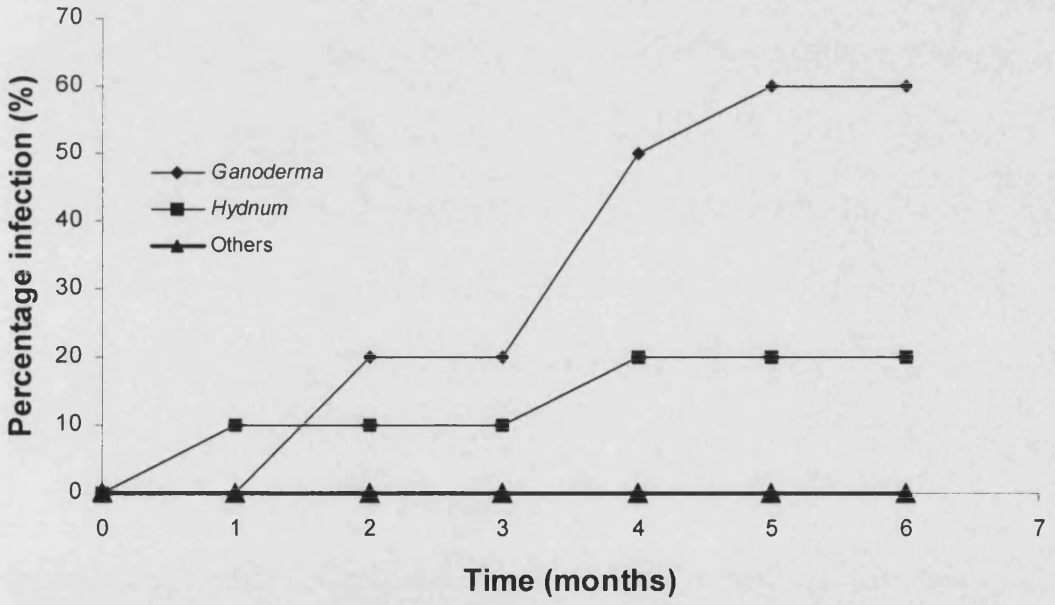

FIG. 75. Infection of seedlings inoculated with degradative fungi and *G. boninense*. No infection was observed from any of the palms inoculated with degraders except *Hydnum* sp. after 6 months.

After six months, 2 of the 10 seedlings exposed to *Hydnum* sp. had died. However, although 2 seedlings were lost from this treatment *Hydnum* could not be re-isolated. The dead tissue did not appear to be infected with a fungus, but extensive bacterial colonisation of the tissue was observed. Mechanical damage by *Oryctes rhinoceros* is common in palm seedlings in Sumatra inducing losses both in the plantation at re-

plant and all palms used in this study showed varying degrees of damage in initial stages. No other palms treated with degraders showed any signs of infection, whilst 60% of control seedlings inoculated with *Ganoderma* showed foliar symptoms of infection or were dead after six months (Fig. 75).

4.3.14 Effect of Temperature on Growth of Potential Biocontrol Fungi

Any BCAs considered for application to windrows must be resistant to extremes of temperature, therefore culture profiles over a range of temperatures was collected for potential antagonistic and degrader fungi. Hyphal extension of the most promising antagonistic *Trichoderma* isolate (SBJ8) and several degrader basidiomycete fungi were tested on PDA at various temperatures (Fig. 76).

Trichoderma SBJ8 had the greatest radial growth rate and this was highest at 30°C; however, the isolate failed to grow at temperatures above 35°C and is therefore unlikely to be able to withstand temperatures in direct sunlight in South East Asia although it remains viable due to production of conidia.

The basidiomycetes grew more slowly than *Trichoderma* sp. with optimum growth typically around 30-35°C. *P. sanguinensis* was the most resistant to elevated temperatures, its optimum temperature was 40°C and hyphal growth at 45°C was comparable with that at lower temperatures. *Trametes hirsuta* also has an optimum temperature of 40°C but showed little or no growth at 45°C. The polypore BLRS7 grew optimally at 35°C but it has a narrow temperature range and growth is considerably reduced at temperatures above and below 35°C. At 45°C there was almost no growth at all and is considerably less thermo-tolerant than *P. sanguinensis*. *Hydnum* sp. displayed a lower optimum temperature of 30°C and did not grow well at 45°C.

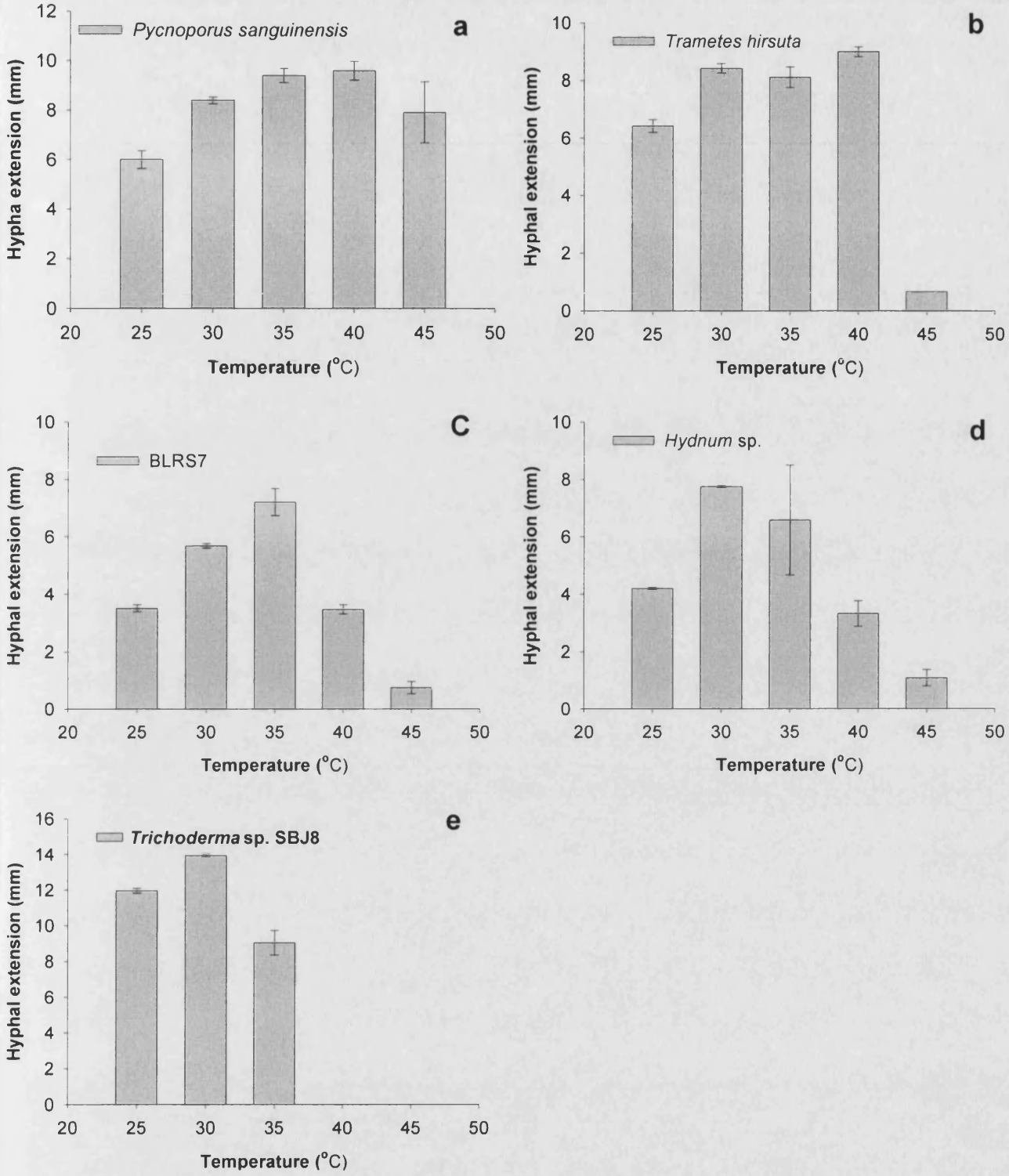

FIG. 76a-e. Hyphal extension of several potential biocontrol fungi at 25-45°C. Fungi were cultured on PDA with three replicates for each temperature. Measurements were made over 10 days except for *T. harzianum*, which was measured over a two-day period because of rapid radial growth.

4.4 Discussion

Fungal species such as *Trichoderma* and *Gliocladium* spp. have been investigated as biocontrol agents alongside common soil bacteria such as *Bacillus* and *Pseudomonas* spp. Isolates from these species have been shown to be inhibitory to numerous plant pathogens including *Rhizoctonia solani*, *Pythium ultimum* and *Sclerotium rolfsii* (216). Over the course of this research, it has become evident that *G. boninense* is not a robust competitor and is unlikely to survive independently in the soil. *G. boninense* is primarily a wood-degrading basidiomycete and is present in standing and toppled palms or in small wood fragments in the field. It exists as metabolically active mycelia or as melanised, thick cell walled resting structures; however *G. boninense* does not appear to form chlamydospores. Success of soil application of BCAs against BSR is likely to be difficult for several reasons: i) infection by *G. boninense* is usually not observed within the first few years after planting, so application of BCAs to the soil at replant may be an inefficient use of time and resources, ii) since *G. boninense* is not found independently in the soil, it could be argued that applications here are unlikely to affect its survival or pathogenicity, and iii) since *G. boninense* is primarily a wood-degrading basidiomycete and is present within palm tissues, application of antagonistic microorganisms to the rhizosphere would not tackle the main source of inoculum, decaying palm wood.

Research into biocontrol of BSR in South East Asia has so far been limited. Inadequate knowledge of pathogenicity and conjecture over epidemiology has made it difficult to ascertain a strategy for use of BCAs against *G. boninense*. Samiyappan *et al* (192) state that *G. boninense* is soil borne and most research to date has followed this assumption, with the result that much of the work on biocontrol of BSR has involved isolation and investigation of antagonistic microorganisms from the oil palm rhizosphere (55, 197, 217). Susanto *et al.* (217) screened one hundred and forty fungal and bacterial isolates from the oil palm rhizosphere for ability to inhibit *G. boninense* colonisation of PDA plates and infection of oil palm seedlings. *Trichoderma harzianum* and *Gliocladium viridae* appeared to be the most aggressive inhibitors of *G. boninense* as no seedlings became infected when treated with these mycoparasites. However, the period of observation was only one year and infection was low in all treatments with only 18% in control seedlings, which suggests the

method of infection was not favourable to the pathogen and that the period of observation may not have been long enough to draw confident conclusions. Sariah *et al* (197) also favour soil application of BCAs and they quantified *Trichoderma* spp. within the A1 (0-30 cm) and Be (30-60 cm) soil horizons of heavily infected and healthy mature oil palm plots. They suggest that soil augmentation with antagonistic *Trichoderma* spp. could be applied at replant and periodically thereafter in an effort to obtain conditions similar to those in suppressive soils.

Suppressive soils are soils where disease development in or on the susceptible host is suppressed, even though the pathogen is present (36). Under field conditions, where suppressive soils have been observed to reduce incidence of disease, monoculture of the host and several years are often required to establish suppressiveness (249). Sariah *et al* (197) investigated levels of *Trichoderma* in soil from oil palm plantations and found that *Trichoderma* spp. were found to be present in higher numbers in a heavily infected plot than in healthy plots, suggesting that presence of potentially antagonistic *Trichoderma* spp. may not aid development of soils suppressive to *G. boninense*. This may be due to the fact that the presumption the *G. boninense* is soil borne is not substantiated and *G. boninense* was shown to be unable to grow in un-sterilised soil or organic field debris in this study (2.3.11). Therefore, targeting BCAs for soil application may not prove successful for tackling the main sources of infection unless organisms can associate with the palm roots over long periods, are extremely antagonistic to *G. boninense* or can induce systemic resistance (ISR) in the palms. The principal problem within oil palm monoculture is *G. boninense* inoculum levels within decaying wood from diseased palms during the productive life and waste material within windrows. A reduction in disease inoculum should reduce incidence of infection and potential BCAs can tackle this in two ways: direct antagonism and competition.

Antagonistic fungi were obtained using the pre-colonised plate technique, which served as an initial screen for efficacy against *G. boninense* and has been used to select for mycoparasites of the plant pathogens *Rhizoctonia solani* and *Botrytis cinerea* (152), *Fusarium pallidoroseum* which causes crown rot of banana (118) and the cocoa pathogens, *Moniliophthora roreri* and *Phytophthora palmivora* (226). Potential mycoparasitic fungi such as *Trichoderma*, *Fusarium*, and *Gliocladium* spp.

are efficient saprophytes and random sampling of these organisms from the soil or leaf litter can result in isolation of a vast number of species (197). In contrast, isolation of antagonists using the pre-colonised plate method selected only for competitive or antagonistic organisms against *Ganoderma*. Subsequent to isolation, preliminary evaluation of antagonists was made with dual cultures. This revealed the advantage of selecting for aggressive isolates at the isolation stage, as isolates obtained from Sumatra were more aggressive against *G. boninense* in dual culture than mycoparasites obtained from culture collections. In absence of an adequate selective medium, some researchers have measured the effect of antagonists in dual culture by the difference in host mycelial radius compared to controls (217). Reduction of host radius can be attributed to contact-mediated antagonism or diffusible inhibitors such as antibiotics. However, limitations of this methodology were observed, as many of the *Trichoderma* isolates grew rapidly and it was impossible to determine if reduced radial growth was a result of diffusible inhibitors or parasitism. Following contact, *Ganoderma* mycelium usually did not extend further and this is probably more a result of competition than parasitism. Many of the organisms initially appeared to be antagonistic towards *Ganoderma*, but when recovery of *Ganoderma* was attempted using GSM, recovery was often easily achieved.

Preliminary screening allowed selection of several *Trichoderma* spp. that appeared to be promising antagonists of *Ganoderma*. When conidia were applied to wood blocks, all of the isolates had the ability to colonise oil palm *in vitro* and enzymatic degradation resulted in approximately 10% dry weight loss. After six weeks the *Trichoderma* isolates remained viable and thus displayed ability to survive on oil palm wood under controlled conditions. Spore suspensions were also inoculated to blocks pre-colonised with *G. boninense* BLRS1. Each of the *Trichoderma* isolates tested resulted in significant reduction in weight loss compared to controls. Characteristic green mycelium covered all the *Trichoderma* treated blocks, though there was considerable variation in recovery of *Ganoderma* between treatments. *Trichoderma* SBJ8 and SBJ10 were very inhibitory to *Ganoderma*; no re-isolation was possible from the surface of the blocks and only 50% of the blocks treated with SBJ10 allowed recovery from inner tissue. Four *Trichoderma* isolates that appeared to be the most inhibitory were then tested against a further two *G. boninense* isolates.

This showed that three of the four *Trichoderma* spp. tested (SBJ8, SBJ10 and BLRS6) were extremely inhibitory to *Ganoderma* wood degradation and the pathogen could not be re-isolated from almost all treated blocks.

The potential of these antagonists to grow independently on oil palm wood and to severely inhibit *G. boninense* colonisation is encouraging for use as inhibitors on wood tissue *in vivo*. However, it was important to determine if disruption of *Ganoderma* growth on wood substrates could interfere with ability to cause disease. This was investigated using two inhibition experiments: 1) Direct application of conidial suspensions to *G. boninense* colonised blocks, before attachment to roots, and 2) Application of fungal spores to the rhizosphere of oil palm seedlings two weeks before addition of *Ganoderma* colonised wood blocks to palm roots. Application of antagonists directly to the inoculum source had greater efficacy at reducing infection than when applied to the rhizosphere. Analysis of inoculum blocks after two months in the soil showed that when SBJ8 and SBJ10 were applied to the wood blocks no viable *Ganoderma* remained. Observation of control blocks showed the surface was covered with tough, melanised hyphae after the same period, indicating that weight loss from test blocks was due to antagonistic activity by the mycoparasites. From the results of these experiments it appears that direct application of antagonistic fungi to inoculum sources may be more effective at reducing infections than application by soil augmentation.

However, the *Trichoderma* isolate SBJ8 may also have potential for soil or seed application. SBJ8 significantly inhibited infection in oil palm seedlings after soil drench compared with controls. Visualisation of *Ganoderma* colonised blocks used to inoculate palm seedlings whose roots had been drenched with a spore suspension of SBJ8 revealed absence of *Ganoderma* mycelia from the surface of almost all blocks after two months in the soil. This is presumably because of antagonistc activity of SBJ8, which remained viable in the soil, under glasshouse conditions, for 23 weeks and viable cells exceeded $2x10^6$ per gram for over ten weeks. Microscopy of root epidermis and outer cell layers from palms exposed to SBJ8 soil drench showed widespread colonisation of the epidermis and on some occasions evidence of penetration into the epidermis and the cells below. Root colonisation by plant growth promoting rhizobacteria (PGPR) (216), non-pathogenic isolates of *Fusarium*

oxysporum (79) and *Trichoderma* spp. has been demonstrated and is implicated in stimulating plant defence responses (95).

Beneficial effects of plant growth promoting rhizobacteria are well established and association of *Pseudomonas aeruginosa* with the roots of chickpea was shown to induce PR proteins including phenolics and resulted in reduced infection rates on subsequent challenge with the pathogen *Sclerotium rolfsii* compared to controls (208). Induced resistance of plants against pathogenic *Fusarium oxysporum* isolates has also been demonstrated and microscopy reveals active colonisation of the epidermis and hypodermis cell layers of tomato roots by non-pathogenic isolates of *Fusarium oxysporum* (Fo47 and Cs-20). Resistance is postulated to be a consequence of a number of factors including: direct antagonism and competition resulting in niche exclusion of the pathogen (79), enzymatic changes in the plant leading to cell wall thickening and intercellular plugging that is more intense in the case of the non-pathogen (23) and increased activity of defence related enzymes such as chitinase, β 1-3 glucanase and β 1-4 glucosidase (81).

Recently it has become known that *Trichoderma* species can form robust and long lasting colonisations of root surfaces and penetrate into the epidermis of plants, inducing localised or systemic resistance responses in the plant (95). *Trichoderma harzianum* T-203 has been found to closely associate with cucumber roots and these interactions were observed to induce production of PR proteins and enzymes involved in host systemic resistance responses (257). Increased activities of peroxidase and chitinase were observed in both roots and leaves of *T. harzianum* T-203 treated plants 48 h post-inoculation. Increased enzymatic activity in both the roots and leaves suggests induction of a systemic response to the presence of *Trichoderma* in the rhizosphere. *T. harzianum* T-22 was also shown to have an effect on plant metabolic activity. Two-dimensional gel electrophoresis of maize treated with *T. harzianum* T-22 showed that 40% of the proteins that were seen in the presence of T-22 were not visible in gels that contained proteins from untreated plants (96). These results suggest that association of BCAs with plant roots may prime plant defence responses and induce a heightened state of resistance in some plants. These responses may also involve structural changes. Ultrastructural studies have revealed that fungal ingress within root epidermis stimulated depostion of callose-rich cell wall appositions onto

the inner cell surface (202). Deposition of callose is known to be part of the plant defence response in numerous plants yet ultrastructure studies have demonstrated this to be part of the response in compatible interactions between Arabidopsis and the downy mildew pathogen *Peronospora parasitica* (212). Microscopy also revealed that invading hyphae were coated in electron dense osmiophilic substances; suggestive of the presence of phenolic compounds known to stain densely upon reaction of *O*-dihydroxy groups with osmium tetroxide (202). Although induction of ISR has not been established for SBJ8 in oil palm, light microscopy showed colonisation of the epidermal cell layer reminiscent of colonisation of plant roots by *T. harzianum* T-203 and it is possible that the success of SBJ8 at reducing root infection may be effected by a combination of its parasitic ability towards *Ganoderma* and priming of host defence responses.

The potent fungitoxic compounds produced by the temperate *Streptomyces* spp. are also promising and these organisms have so far been overlooked in BSR biocontrol studies. *Streptomyces griseoviridis* was used in this study and is marketed as Mycostop® (Verdara) and is a BCA for several soil-associated plant pathogens including: *R. solani* which causes damping off of cauliflower, *Pythium* spp. that cause disease in a number of plants including greenhouse cucumber, and *Alternaria* spp. that cause damping-off (reduced germination and emergence of seedlings) in cabbage and carrot cultivations (222, 243). *Streptomyces* spp. are known to produce many anti-bacterial and anti-fungal compounds (38) and the success of *S. griseoviridis* at inhibiting plant pathogens is attributed to production of these compounds. Although data from this study are preliminary, there are two potential applications of actinomycetes as BCAs in oil palm plantations.

The first potential application would be addition of *Streptomyces* isolates either alone or in combination with other BCAs, to encourage degradation of oil palm debris and induce conditions inhibitory to *Ganoderma* colonisation. This would require application of new land preparation methods involving pulverisation of palm material. Reduction of palm wood to small fragments and spreading of the wood chips throughout fields might produce conditions favourable to the development of *Streptomyces* spp. that can produce lignocellulose-degrading enzymes, whilst antibiosis could preclude colonisation by *G. boninense*. Ooi and Heriansyah (160)

report that by shredding oil palm tissue and spreading the wood-chips over the soil, loss of dry matter can be as high as 80% in 56 weeks. This may provide an opportunity for application of antibiotic producing actinomycetes such as *Streptomyces viridosporus* strain T7A that can produce LiP and proliferate in lignocellulose-rich environments (257) and form the basis of a soil augmentation strategy in an integrated management practice (IMP).

When tested for activity against *G. boninense* in dual culture, *S. griseoviridis* produced diffusible inhibitors which created an inhibition zone >1 cm, which separated the cultures and was maintained for over two weeks. It could also inhibit *Ganoderma* colonisation of wood blocks; addition of a *Streptomyces* suspension supplemented with glucose one week before inoculation of *Ganoderma* inhibited successful colonisation by the fungus. However, addition of *Streptomyces* without supplementation with glucose, or concurrently with *Ganoderma* did not result in the same inhibition. This suggests that build up of antibiotics was responsible for the inhibition and that glucose was required as an energy source. Some *Streptomyces* spp. have been shown to be able to utilise lignin, *Streptomyces viridosporus* strain T7A is found in leaf litter and production of LiP requires glycerol supplementation to induce lignocellulose degradation in liquid culture (102, 258). Increased inhibitory ability of *S. griseoviridis* on wood blocks when supplemented with glucose perhaps facilitates the production of lignocellulose degrading enzymes and colonisation of the blocks.

The second application would be direct application to the soil as part of an integrated management policy. A prerequisite for any potential soil applied BCA would be a proven ability to associate with the root surface of oil palm and production of anti-fungal compounds or induction of host defence responses. Root colonisation of carrot (*Daucus carota*) and turnip rape (*Brassica rapa* subsp. *oleifera*) by *S. griseoviridis* has also been demonstrated (117). Over a three-week period there was no evidence of plant growth promoting effects from the association, however the sample period was short and the benefits of root colonisation by the BCA *S. griseoviridis* may only emerge when plants are challenged by pathogens. The ability of *Streptomyces* to colonise turnip roots was much greater than those of carrots, showing that root colonisation varies between plants. The potential benefits of root colonising bacteria are displayed in the interaction between *P. aeruginosa* and the roots of groundnut

(*Arachis hypogaea*). Presence of *P. aeruginosa* within the rhizosphere of groundnut was shown to inhibit *Sclerotium rolfsii* stem rot mortality by 54%-58% (116). Cell free culture filtrates of the bacteria were shown to inhibit CWDE production by the pathogen and this was proposed as one of the factors responsible for inhibition.

Since CWDEs are thought to be critical for invasion of oil palm and association of bacterial and fungal species with roots of other plants has been shown to induce plant resistance responses, a suspension of *S. griseoviridis* was applied to the rhizosphere of oil palm seedlings two weeks before pathogen inoculation to test for inhibition. Unfortunately *S. griseoviridis* was ineffective at reducing disease incidence; this may be due to the organism multiplying in the soil but not associating with the root surface, or perhaps there was inadequate time to build up the anti-fungal conditions in the rhizosphere. Therefore application of this isolate would perhaps be most promising as part of a palm pulverisation strategy at replant rather than as a means of attaining ISR by close association with palm roots over long periods.

However, even if inhibition of infection were successful, *S. griseoviridis* is a temperate species and would be unlikely to thrive in tropical conditions alongside other locally adapted microorganisms. Furthermore, introduction of foreign organisms, accidental or deliberate, to new habitats is perilous and can result in the development new problems. For instance, introduction of the corn rootworm (*Diabrotica vigifera*) to Eastern Europe from the United States was first identified outside Belgrade, but has since spread to Hungary and Croatia and is expected eventually to spread to all corn producing nations of Europe (69). Therefore care should be taken when considering release of non-native BCAs to the environment. Nevertheless, the anti-fungal compounds produced by *S. griseoviridis* are of interest and highlight the inhibitory potential of actinomycetes. A local screen could be undertaken to unearth native isolates inhibitory to *G. boninense* using *Streptomyces*-selective medium (120).

Nevertheless, the most pressing concern within oil palm cropping systems is the need to reduce levels of *G. boninense* inoculum and this may be a difficult task since the pathogen is clearly well adapted to decay of palm wood. Additionally, burning of palm wood has been banned in South East Asia because of associated smog problems,

resulting in vast amounts of palm wood waste within plantations. Ensuing production of large quantities of basidiospores is probably responsible for increasing levels of USR of palms in neighbouring blocks; when these palms topple they become infection foci within plantings, resulting in increased incidence of BSR. Reducing inoculum in wood debris is a daunting task for control, and there is so far no BCA or chemical control measure to tackle this. Addition of BCAs to palm trunks early after toppling may encourage colonisation of non-infected tissue by competitive, but non-pathogenic fungi, thus reducing the potential inoculum for neighbouring palms. Pre-emptive addition of BCAs is also an important strategy in attempts to control cocoa pod diseases using endophytic mycoparasites (119, 226) and biocontrol of pine root rot in temperate regions is achieved by application of a spore suspension of the basidiomycete, *P. gigantea* (Rotstop®) to cut stumps of pine trees after felling (243). Colonisation by this fungus stops the pathogen (*H. annosum*) from colonising and therefore denies it access to the dead roots from where infection of standing trees occurs. Exclusion of the pathogen from potential reservoirs would be the best strategy for using wood degrading fungi as BCAs.

Wood degrading basidiomycetes were tested for ability to induce weight loss of oil palm blocks *in vitro*. Although all organisms tested were isolated from decaying palm wood, several failed to establish under laboratory conditions. Paterson *et al* (163) had similar experiences when investigating usefulness of wood degrading basidiomycetes at breaking down oil palm wood in vitro. They found that certain isolates were unable to establish on oil palm blocks *in vitro* and postulated that this was because of inadequate inoculation. However, degradation of wood in the environment is a sequential process and often one fungus initially colonises tissue only to be ousted later by another fungus in a process termed "succession" (181). It is possible that these organisms do not grow well on virgin oil palm wood, but instead colonise after a certain level of decay has already occurred.

The most effective degraders of oil palm wood in the lab were further investigated for ability to decay oil palm tissue under field conditions. However, none of the fungi induced significantly greater degradation of oil palm wood than controls. Nevertheless, no *G. boninense* basidiophores were observed suggesting that *Ganoderma* was unable to establish in treated wood, at least to the point of producing

basidiophores. This degree of limitation may be sufficient in terms of controlling spread within plantations.

If degradative fungi were to be used in the field, culture characteristics of the fungi at various temperatures suggests that *P. sanguinensis* and *Trametes hirsuta* are thermo-tolerant and could be considered for application to windrows, whereas the polyporous isolate BLRS7 and the *Hydnum* sp. would likely have greater efficacy in shaded conditions. Degradation of wood blocks by *P. sanguinensis* and BLRS7 were tested biochemically and were shown to be simultaneous degraders of oil palm wood. In particular, *P. sanguinensis* was apparent as a rapid coloniser of oil palm wood *in vitro* and this feature, added to its thermo-tolerance, are promising for colonisation of tissue under field conditions.

The main inoculum source of *Ganoderma* within plantations is in decaying wood tissue and these remain infectious for at least two years (99). Treatment of whole windrows may be too ambitious for the purposes of biocontrol because of the amount of material (*ca.* 85t/ha) involved; also BCAs released on windrows may be exposed to high levels of ultraviolet radiation and fluctuating temperatures that can be inhibitory to fungi (186). A more realistic target for biocontrol would be palms that topple midway through their productive life. Currently these palms are left where they fall; the only measure taken to reduce risk of infection to neighbouring palms is removal of the bole from the soil. These become a potentially dangerous source of infection, and roots from neighbouring palms are readily observed protruding into decaying tissue. Canopy closure maintains optimal temperatures for *G. boninense* proliferation, and shading has been shown to markedly enhance infectivity in seedling trials (see chapter 1). Conversely, under these conditions, BCAs would also be relatively protected from UV and marked changes in relative humidity and temperature. Cost of removal may be prohibitive, so application of a cocktail of BCAs may be useful for reducing inoculum potential. Wood-degrading basidiomycetes could be inoculated to un-colonised wood tissue to reduce the nutrient source available for the pathogen and antagonistic fungi such as *Trichoderma* SBJ8 could be applied to colonised tissue to disrupt colonisation and parasitise the pathogen. However all isolates shown to be antagonistic to *G. boninense* were also shown to be inhibitory to other basidiomycetes (data not shown).

Another possible application of antagonistic BCAs is to cut fronds and peduncles. If USR occurs as a result of germination of basidiospores on wound surfaces, application of antagonists to cut fronds or peduncles may reduce USR incidence. However, the challenge of application to cut fronds and peduncles in mature palms would be problematic. Harvesting of fruit bunches from mature oil palms results in numerous cut fronds and peduncles at elevation, which poses problems for delivery of BCAs. If this proved to be prohibitive, it would probably not be sensible to employ a strategy of treating lower wound surfaces and not those that are more elevated. Furthermore, logistical considerations may also preclude this strategy; the number of wounds inflicted on palms during harvesting would require application of antagonistic mixtures on a continuous basis. This would require either the harvester to be trained in handling and application of BCAs, which would reduce their harvesting efficiency, or additional personnel would need to be trained, which would increase financial costs. Given that infection of upper stems is presumed to be as a result of relatively infrequent events and route of infection has still not been satisfactorily established, expenditure of time and resources on this method of disease management would not be recommended in light of the uncertainty surrounding USR infection.

No single BCA is likely to possess all attributes required to affect control under field conditions. For instance, isolates that produce enzymes or antibiotics that are associated with biocontrol may not be able to function well at temperatures and moisture levels where the pathogens flourish (105). This has been borne out in this study as some isolates have been shown to be promising degraders of oil palm, but are not thermotolerant, therefore limiting their likely efficacy on windrows. In addition, none of the biodegradative isolates appeared to be antagonistic to *G. boninense* and all were inhibited by *Trichoderma* isolates shown to be antagonistic to *Ganoderma*, precluding a mixed inoculum containing both antagonists and biodegraders.

Overall the studies suggest that the most promising fungus for control of BSR unearthed during this study is the *Trichoderma* isolate SBJ8. This isolate can survive in wood and in the soil under controlled conditions and when applied as a spore suspension to *Ganoderma* mycelium on PDA plates or wood blocks, complete destruction of *Ganoderma* mycelium usually occurs. SBJ8 merits further study to determine if promising laboratory results can be replicated under field conditions.

Inhibition assays should be conducted using a variety block sizes to determine if efficacy can be maintained when there is a large inoculum. Soil persistence trials could also be conducted to determine longevity in various plantation soils. This would require soil to be heat-treated to kill indigenous *Trichoderma* spp. and careful control of conditions to ensure minimal contamination of soil from external sources since TSM will facilitate isolation of many species of *Trichoderma.*

5 Conclusions and Future Work

In this study *G. boninense* was shown to be a weak competitor and was unable to grow in non-sterilised soil or frond debris. Therefore it is unlikely that *Ganoderma* stem rot infections occur directly from isolates living saprophytically in the soil or from colonised organic matter that collects behind the frond axils. However, the possibility that basidiospores may infect upper stems directly cannot be discounted. This study showed for the first time that basidiospores from *G. boninense* are capable of germinating on trunk, frond and peduncle wounds. Substantial numbers of other fungal species and bacteria were observed on the wound surfaces and these may potentially inhibit establishment of *G. boninense* in many instances. However, it is possible that basidiospores may be drawn into xylem vessels to a depth of possibly 10 cm if they land immediately after wounding, which may reduce the number of competitors on germination. Direct infection by basidiospores is also likely to require anastomosis as no successful infection by monokaryons has yet been reported and dikaryotic mycelium was shown to be necessary for infection of roots in this study.

Root infection remains the likeliest means of establishment of BSR infections and root infection was clearly demonstrated in this study. Although generally accepted as the main route of infection in oil palm, root infection under natural conditions has still not been conclusively proven. However, infection studies by Hasan and Turner (99) using bait seedlings placed around infected mature palms, and more recent work by Flood and Hasan (78) where seedlings placed around buried palm trunks rapidly became infected by *G. boninense* are strongly indicative of root infection. Findings from this project suggest that windrows may not be the greatest source of *G. boninense* infection in plantations. Hasan and Turner (99) showed that felled trunks were only infectious for approximately two years, whereas BSR infections usually begin to be observed after about eight years after planting. There is no reason to suspect that there is a protracted incubation time before disease symptoms and it was postulated that from infection to foliar symptoms would take two years in mature oil palm (180). Additionally, where land preparation has been poor and boles have not been removed from the soil, high losses in juvenile palms can occur (Hugh Foster, pers. comm).

If root infection is accepted as the basis for high levels of BSR infections, evidence from this study suggests that fallen palms (FPs) may potentially represent the most likely source of inoculum during the planting cycle. USR infection as a result of infection by basidiospores may result in isolated tree mortalities; these trees then topple and become infection foci for neighbouring palms. Canopy closure is likely to create conditions favourable to *G. boninense*; in this study, *G. boninense* was shown to grow extremely well in culture at 25-30°C and in Sumatra temperature extremes were minimal in the soil of shaded seedlings, not exceeding 32°C. Basidiospores would be likely to land and germinate on these toppled palms, and this could result in rapid and complete colonisation of the trunks with multiple genetically distinct individuals. Use of a centrifugal air sampler in Sumatra showed that there was *ca.* 3000 *Ganoderma* basidiospores/m^3 of air in an 8 year old plot and almost 5000 basidiospores/m^3 air in 17 year old plots. In the presence of such high numbers of spores, it seems likely that numerous isolates would be present on dead palm material and additionally, such large numbers would increase the potential for the conditions under which basidiospores can progress from simply germinating on wound surfaces to development of infection. However, the weak competitive ability of *G. boninense* in other natural plantation niches (soil, frond debris) must question the frequency of this mode of initial establishment.

Genetic fingerprinting of isolates within FPs and adjacent BSR infected palms was unable to associate any of adjacent infections to isolates within FPs; this may be as a result of the likely high number of genetically distinct isolates within FPs as a result of colonisation by basidiospores. In addition, if it takes as long as two years from initial infection to foliar symptoms or basidiophore development (180), isolates present within the FPs years earlier may have been succeeded during the intervening period. However, two adjacent BSR infected palms did share RAMS profiles; BSR BC B5 S2 had an identical band pattern to BSR BD B3 S2 in plot 86:400 from Sungei Bejanker, indicating that both trees contacted the same inoculum source or that root-root contact was responsible for transmission of the disease. Probable evidence of vegetative infection from plantations in Malaysia was also brought to light by Miller *et al* who recorded similar findings (142) where adjacent BSR infected palms, 102SB and 103SB, contained the same mtDNA RFLP band pattern.

Nevertheless, identification of only two adjacent palms infected with genetically identical individuals is not sufficient to prove the basis of root infection beyond doubt. To study this issue conclusively would take several years and would necessitate identification of USR or isolated BSR infections within a young stand of oil palm. Isolates should be collected from basidiophores immediately as the infected palms topple and at intervals thereafter (e.g. every 4 months collecting every fruiting body). RAMS profiles should be made and each distinct profile recorded, with cultures stored under oil on agar slants for later reference. All palms within four planting rows of FPs should be examined for BSR symptoms and isolates collected periodically from basal fruiting bodies. In this way, a genetic profile of all isolates present within the vicinity over several years could be built up and determination of source of infection could be established. However, if no identical RAMS banding patterns were found within BSR palms and FPs, this would be strong evidence against vegetative spread of the disease.

This study has shown that *G. boninense* appears well adapted to growth on oil palm. Enzymatic activity from liquid culture and extracts from decaying wood blocks confirms that *G. boninense* produces a complete array of lignocellulose degrading enzymes that allow it to efficiently colonise and degrade oil palm wood. Ultrastructure revealed that cell wall degrading enzymes (CWDE) are likely to be important during infection and it is possible that some of these are virulence determinants. The most aggressive isolate in this study was *G. boninense* GMR3 and this was capable of infecting non-wounded roots with the same efficacy as wounded roots; less aggressive isolates have significantly reduced ability to infect intact roots. Penetration of lignified and suberised outer barrier layers would require production of corresponding CWDEs and these are potential virulence determinants. As yet virulence determinants in *G. boninense* have not been elucidated and no studies have been conducted to address this issue, however, production of extracellular laccase may be a possible candidate for preliminary investigation. Application of techniques to monitor gene expression during key stages of infection and colonisation may be the method of choice for infection of oil palm by *G. boninense* and other little understood root/wood invading basidiomycetes.

Biocontrol of *Ganoderma* stem rot is a possibility and this study has identified numerous candidate antagonistic fungi. The most promising isolate was *Trichoderma* sp. SBJ8, which was shown to be highly antagonistic to *Ganoderma* when applied directly to colonised wood blocks and was also able to reduce infection of seedlings after soil drench. Microscopy reveals that this isolate can colonise the epidermal and outer cortex layers of oil palm roots. In other plants, colonisation of outer cell layers by *Trichoderma* spp. has been shown to prime defence responses; reduced infection of oil palm after application to the rhizosphere may be due to a combination of its antagonistic abilities against *G. boninense* and induction of defence responses. Actinomycetes may also be useful candidates for biocontrol and *Streptomyces griseoviridis* was shown to produce compounds inhibitory to *G. boninense* in this study. Numerous biodegradative basidiomycete fungi were also screened for ability to colonise and decay oil palm tissue, and the most prominent of these were *Pycnoporus sanguinensis* and an unidentified polyporous isolate, BLRS7. However, none of the isolates tested were able to induce more rapid and extensive degradation of large oil palm discs than uninoculated controls under natural conditions.

Land preparation at replant is undoubtedly crucial and further investigation into the feasibility of implementing shredding or pulverising oil palm trunks could prove beneficial. Comminuting trunks into fine debris would reduce the number of *G. boninense* basidiophores due to the absence of windrows; also actinomycetes that are inhibitory to *Ganoderma* could be applied to the debris as it is spread across the field. Actinomycetes have been shown to grow well in leaf litter and compost, and some isolates can produce lignin-degrading enzymes, which may enable them to colonise shredded oil palm material. However, care must be taken here as it is uncertain how long *G. boninense* can survive in palm debris and whether such small inoculum is sufficient to infect seedlings. Nevertheless, application of this method and concurrent application of BCAs antagonistic to *G. boninense* should minimise production of basidiophores, which are responsible for the high levels of basidiospores in plantations and therefore, potentially, the increasing incidence of USR.

To tackle the problem of BSR during the productive life of a plantation, the problem of fallen palms left in the field must be addressed. They rapidly become colonised by *G. boninense*, with associated production of basidiophores and roots from

neighbouring trees can often be seen extending into these trunks on the soil surface, which probably leads to BSR infections. Ideally, these palms, and all bole material, would be removed as they fall to nearby windrows or for shredding. However, if this is not financially viable, immediate application of *Trichoderma* sp. SBJ8 or other antagonistic fungi, localised to the area of *G. boninense* colonised tissue, could be used to promote active destruction of the pathogen. Concurrent application of non-pathogenic basidiomycetes along the non-colonised points on the trunk should also be undertaken to promote degradation of the material by non-pathogenic species and preclude *Ganoderma* from large portions of the material by niche exclusion.

Resistance to *G. boninense* remains the ideal means of disease control and the facile method described in this study, involving challenging roots with small inoculum sources under shaded field conditions should enable screening of palm crosses for resistance. Also, the method enables distinguishing between levels of aggressiveness of *G. boninense* isolates; this information will be crucial for choosing one or more isolates for a representative screening programme.

References

1. **Abadi, A. L.** 1990. Antagonistic effect of four fungal isolates to *Ganoderma boninense*, the causal agent of Basal Stem Rot of oil palm. Biotropia **3:**41-49.
2. **Adaskaveg, J. E., R. A. Blanchette, and R. L. Gilbertson.** 1991. Decay of date palm wood by white-rot and brown-rot fungi. Canadian Journal of Botany-Revue Canadienne De Botanique **69:**615-629.
3. **Adaskaveg, J. E., and R. L. Gilbertson.** 1988. Basidiospores, Pilocystidia, and other basidiocarp characters in several species of the *Ganoderma lucidum* complex. Mycologia **80:**493-507.
4. **Adaskaveg, J. E., and R. L. Gilbertson.** 1986. Cultural studies and genetics of sexuality of *Ganoderma lucidum* and *G. tsugae* in relation to the taxonomy of the *G. lucidum* complex. Mycologia **78:**694-705.
5. **Adaskaveg, J. E., and R. L. Gilbertson.** 1989. Cultural studies of four North American species in the *Ganoderma lucidum* complex with comparisons to *G. lucidum* and *G. tsugae*. Mycological Research **92:**182-191.
6. **Adaskaveg, J. E., and R. L. Gilbertson.** 1995. Wood decay caused by *Ganoderma* species in the *G. lucidum* complex, p. 79-93. *In* P. K. Buchanan, R. S. Hseu, and J. M. Moncalvo (ed.), *Ganoderma*: Systematics, Phytopathology and Pharmacology. Proceedings of Contributed Symposium 59, A,B, 5th International Mycological Congress. Taipei. Pp., Vancouver.
7. **Adaskaveg, J. E., R. L. Gilbertson, and R. A. Blanchette.** 1990. Comparative-studies of delignification caused by *Ganoderma* species. Applied and Environmental Microbiology **56:**1932-1943.
8. **Adrian, M., H. Rajaei, P. Jeandet, J. Veneau, and R. Bessis.** 1998. Resveratrol oxidation in *Botrytis cinerea* conidia. Phytopathology **88:**472-476.
9. **Agosin, E., R. A. Blanchette, H. Silva, C. Lapierre, K. R. Cease, R. E. Ibach, A. R. Abad, and P. Muga.** 1990. Characterisation of palo podrigo, a natural process of delignification in wood. Applied and Environmental Microbiology **56:**65-74.
10. **Aist, J. R.** 1976. Papillae and Related Wound Plugs of Plant-Cells. Annual Review of Phytopathology **14:**145-163.
11. **Amey, R. C., P. R. Mills, A. Bailey, and G. D. Foster.** 2003. Investigating the role of a *Verticillium fungicola* beta-1,6-glucanase during infection of *Agaricus bisporus* using targeted gene disruption. Fungal Genetics and Biology **39:**264-275.
12. **Anke, H., Kinn, J., Bergquist, K. and Sterner, O.** 1991. Production of siderophores by strains of the Genus *Trichoderma*. Biological Metals **4:**176-180.
13. **Archibald, F. S.** 1992. A new assay for lignin-type peroxidases employing the dye azure-B. Applied and Environmental Microbiology **58:**3110-3116.
14. **Ariffin, D., A. S. Idris, and G. Singh.** 2000. Status of *Ganoderma* in oil palm, p. 49-68. *In* J. Flood, P. Bridge, and M. Holderness (ed.),

Ganoderma Diseases of Perennial Crops. CABI Publishing, Wallingford.

15. **Ariffin, D., and I. A. Seman.** 1991. A selective medium for the isolation of *Ganoderma* from diseased tissues. Palm Oil Research Institute of Malaysia.
16. **Asiegbu, F. O.** 2000. Adhesion and development of the root rot fungus (*Heterobasidion annosum*) on conifer tissues: effects of spore and host surface constituents. FEMS Microbiology Letters **33:**101-110.
17. **Asiegbu, F. O., A. Adomas, and J. Stenlid.** 2005. Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. s.l. Molecular Plant Pathology **6:**395-409.
18. **Ayres, P. G., G. C. Papivizas, and A. F. Diem.** 1966. Polygalacturonate transeliminase and polygalacturonase production by *Rhizoctonia solani.* Phytopathology **59:**925-930.
19. **Barry, K. M., N. W. Davies, and C. L. Mohammed.** 2001. Identification of hydrolysable tannins in the reaction zone of *Eucalyptus nitens* wood by high performance liquid chromatography-electrospray ionisation mass spectrometry. Phytochemical Analysis **12:**120-127.
20. **Baumgartner, K., and D. M. Rizzo.** 2001. Ecology of *Armillaria* spp. in mixed-hardwood forests of California. Plant Disease **85:**947-951.
21. **Becker, J. O., and R. J. Cook.** 1988. Role of siderophores in suppression of *Pythium* species and production of increased growth response of wheat by fluorescent pseudomonads. Phytopathology **78:**778-782.
22. **Bedford, G. O.** 1980. Biology, Ecology, and Control of Palm Rhinoceros Beetles. Annual Review of Entomology **25:**309-339.
23. **Benhamou, N., and C. Garand.** 2001. Cytological analysis of defense-related mechanisms induced in pea root tissues in response to colonization by nonpathogenic *Fusarium oxysporum* Fo47. Phytopathology **91:**730-740.
24. **Bermingham, S., L. Maltby, and R. C. Cooke.** 1995. A critical assessment of the validity of ergosterol as an indicator of fungal biomass. Mycological Research **99:**479-484.
25. **Bhaskaran, R.** 2000. Management of basal stem rot disease of coconut caused by *Ganoderma lucidum*, p. 121-128. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. Cabi Publishing, Wallingford.
26. **Bhaskaran, R., and Karthikeyan, A.** 1994. A method for assessing severity of basal stem rot disease of coconut. Journal of Plantation Crops **22:**93-96.
27. **Blanchette, R. A.** 1991. Dlignification by wood-decay fungi. Annual Review of Phytopathology **29:**381-398.
28. **Blanchette, R. A.** 1984. Selective delignification of eastern hemlock by *Ganoderma tsugae.* Phytopathology **74:**153-160.
29. **Boddy, L.** 1983. Effect of Temperature and Water Potential on Growth-Rate of Wood-Rotting Basidiomycetes. Transactions of the British Mycological Society **80:**141-149.
30. **Boddy, L.** 1992. Microenvironmental aspects of xylem defenses to wood decay fungi, p. 96-132. *In* R. A. Blanchette and A. R. Biggs (ed.),

Defense Mechanisms of Woody Plants Against Fungi. Springer-Verlag, Berlin.
31. **Breton, F., Y. Hasan, Z. Lubis, and H. de Franqueville.** 2005. Presented at the International palm oil congress (PIPOC), Kuala Lumpur.
32. **Bridge, P. D., E. B. O'Grady, C. A. Pilotti, and F. Sanderson.** 2000. Development of molecular diagnostics for the detection of *Ganoderma* isolates pathogenic to oil palm, p. 225-234. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
33. **Bridge, P. D., G. Panchal, F. Sanderson, and C. Pilotti.** 2001. Presented at the Proceedings of the International Palm Oil Congress.
34. **Burns, C., K. E. Gregory, M. Kirby, M. K. Cheung, M. Riquelme, T. J. Elliott, M. P. Challen, A. Bailey, and G. D. Foster.** 2005. Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns. Fungal Genetics and Biology **42:**191-199.
35. **Campbell, M. M., and P. R. Sederoff.** 1996. Variation in lignin content and composition. Plant Physiology **110:**3-13.
36. **Campbell, R.** 1989. Biological Control of Microbial Plant Pathogens. Cambridge University Press, Cambridge.
37. **Castillo, M. d., S. V. Wiren-Lehr, I. Scheunert, and L. Torstensson.** 2001. Degradation of isoproturon by the white rot fungus *Phanerochaete chrysosporium*. Biology of Fertile Soils **33:**521-528.
38. **Castillo, U. F., G. A. Strobel, and E. J. Ford.** 2002. Munincumbicins, wide-spectrum antibiotics produced by *Streptomyces* NRRL 30562, endophytic on *Kennedia nigriscans*. Microbiology **148:**2675-2685.
39. **Chacha, M., G. Bojase-Moleta, and R. R. T. Majinda.** 2005. Antimicrobial and radical scavenging flavonoids from the stem wood of *Erythrina latissima*. Phytochemistry **66:**99-104.
40. **Christian, J., and G. Lysek.** 1995. Fluorescence microscopy of fungi in native soil - Improvement by additional substances. Microscopy and Analysis **49:**7-9.
41. **Cobb, B. D., and J. M. Clarkson.** 1993. Detection of molecular variation in the insect pathogenic fungus *Metharizium* using RAPD-PCR. FEMS Microbiology Letters **112:**319-324.
42. **Coetzee, M. P. A., B. D. Wingfield, J. Roux, P. W. Crous, S. Denman, and M. J. Wingfield.** 2003. Discovery of two northern hemisphere *Armillaria* species on Proteaceae in South Africa. Plant Pathology **52:**604-612.
43. **Coffey, M. D.** 1975. Obligate parasites of higher plants, particularly rust fungi. Symposia of the Society for Experimental Biology **29:**297-323.
44. **Collins, P. J., J. A. Field, P. Teunissen, and A. D. Dobson.** 1997. Stabilisation of lignin peroxidases in white rot fungi by tryptophan. Applied and Environmental Microbiology **63:**2543-2548.
45. **Cooper, R. M.** 1983. The mechanisms and significance of enzymic degradation of host cell walls, p. 101-135. *In* J. A. Callow (ed.), Biochemical Plant Pathology. John Wiley and Sons Ltd.
46. **Cooper, R. M.** 1984. The role of cell wall-degrading enzymes in infection and damage. *In* R. K. S. Wood and G. J. Jellis (ed.), Plant

Diseases: Infection, Damage and Loss. Blackwell Scientific Publications, Oxford.
47. **Cooper, R. M., and R. K. S. Wood.** 1975. Regulation and synthesis of cell wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici.* Physiological Plant Pathology **5:**135-156.
48. **Corley, R. H. V., and P. B. Tinker.** 2003. The Oil Palm 4th Edition. Blackwell Publishing, Oxford.
49. **Crozier, J., K. Holmes, G. Craig, and S. Nayagam.** 2000. In vitro screening for fungal antagonists of wood decay fungi in tropical hardwoods. Material und Organismen **34:**245-260.
50. **Dai, Y. C., E. J. Vainio, J. Hantula, T. Niemela, and K. Korhonen.** 2003. Investigations on *Heterobasidion annosum* S. lat. in central and eastern Asia with the aid of mating tests and DNA fingerprinting. Forest Pathology **33:**269-286.
51. **Darmono, T. W.** 1998. Development and survival of *Ganoderma* sp. in oil palm tissue. International oil palm conference:613-617.
52. **Darmono, T. W.** 2000. *Ganoderma* in oil palm in Indonesia: Current status and prospective use of antibodies for the detection of infection, p. 249-275. *In* J. Flood, Bridge, P.D., and Holderness, M. (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
53. **de Jong, E., J. A. Field, and J. A. M. de Bont.** 1994. Aryl alcohols in the physiology of ligninolytic fungi. FEMS Microbiology Reviews **13:**153-188.
54. **Deacon, J. W.** 2002. The microbial world: www:helios.bto.ed.ac.uk/bto/microbes/microbes.htm.
55. **Dharmaputra, O. S., H. S. Seomarmi Tjitrosomo, and A. L. Abadi.** 1990. Antagonistic effect of four fungal isolates to *Ganoderma boninense*, the causal agent of basal stem rot of oil palm. Biotropia **3:**41-49.
56. **Dill, I., and G. Kraepelin.** 1986. Palo podrido: model for extensive delignification of wood by *G. applanatum.* Applied and Environmental Microbiology **52:**1305-1312.
57. **d'Souza, T. M., K. Boominathan, and C. A. Reddy.** 1996. Isolation of laccase gene-specific sequences from white rot and brown rot fungi by PCR. Applied and Environmental Microbiology **62:**3739-3744.
58. **d'Souza, T. M., C. S. Merritt, and C. A. Reddy.** 1999. Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum.* Applied and Environmental Microbiology **65:**5307-5313.
59. **Durand-Gasselin, T., H. Asmady, A. Flori, J. C. Jacquemard, Z. Hayun, F. Breton, and H. de Franqueville.** 2005. Possible sources of genetic resistance in oil palm (*Elaeis guineensis* Jacq.) to basal stem rot caused by *Ganoderma boninense* - prospects for future breeding. Mycopathologia **159:**93-100.
60. **Durands, P. K.** 1988. Development and analysis of pectic screening media for use in the detection of pectinase mutants. Applied microbial biotechnology **28:**463-467.
61. **Eaton, R. A., and M. D. C. Hale.** 1993. Wood: Decay, Pests and Protection. Chapman & Hall, London.

62. **Edel, V., C. Steinberg, N. Gautheron, and C. Alabouvette.** 1996. Evaluation of restriction analysis of polymerase chain reaction (PCR)-amplified ribosomal DNA for the identification of *Fusarium* species. Mycological Research **179:**179-187.
63. **Edem, D. O.** 2002. Palm oil: biochemical, physiological, nutritional, haematological, and toxological aspects: a review. Plant Foods for Human Nutrition **57:**319-341.
64. **Effland, M. J.** 1977. Modified procedure to determine acid-Insoluble lignin in wood and pulp. Tappi **60:**143-144.
65. **Eisen, J. A.** 1999. Mechanistic basis for microsatellite instability, p. 34-48. *In* D. B. Goldstein and C. Schlotterer (ed.), Microsatellites. Oxford University Press, New York.
66. **Elad, Y., I. Chet, and Y. Henis.** 1983. Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii*-scanning electron microscopy and fluorescence microscopy. Phytopathology **73:**85-88.
67. **Elad, Y., Z. Sadowsky, and I. Chet.** 1987. Scanning electron microscopical observations of early stages of interaction of *Trichoderma harzianum* and *Rhizoctonia solani*. Transactions of the British Mycological Society **88:**259-263.
68. **Elliot, M. L., T. K. Broschadt, J. Y. Uchida, and G. W. Simone.** 2004. Compendium of Ornamental Palm Diseases and Disorders. American Phytological Society.
69. **Enserink, M.** 1999. Biological invaders sweep in. Science **285:**1834-1836.
70. **Eriksson, K. E., R. A. Blanchette, and P. Ander.** 1990. Microbial and Enzymatic Degradation of Wood and Wood Components. Springer-verlag, Berlin.
71. **Eyles, A., N. W. Davies, T. Mitsunaga, R. Mihara, and C. Mohammed.** 2004. Role of *Eucalyptus globulus* wound wood extractives: evidence of superoxide dismutase-like activity. Forest Pathology **34:**225-232.
72. **Eyles, A., N. W. Davies, and C. Mohammed.** 2003. Novel detection of formylated phloroglucinol compounds (FPCs) in the wound wood of Eucalyptus globulus and E-nitens. Journal of Chemical Ecology **29:**881-898.
73. **Faison, B. D., K. T. Kirk, and R. L. Farrell.** 1986. Role of veratryl alcohol in regulating ligninase activity in *Phanerochaete chrysosporium*. Applied and Environmental Microbiology **52:**251-254.
74. **Faison, B. D., and T. K. Kirk.** 1985. Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. Applied and Environmental Microbiology **49:**299-304.
75. **Farkas, V., M. Liskova, and P. Biely.** 1985. Novel media for detection of microbial producers of cellulase and xylanase. FEMS Microbiology Letters **28:**137-140.
76. **Fisher, J. B., and K. Jayahandran.** 1999. Root structure and arbuscular mycorrhizal colonisation of the palm *Serenoa repens* under field conditions. Plant and Soil **217:**229-241.
77. **Flood, J., S. Hasan, and H. Foster.** 2002. *Ganoderma* diseases of oil palm - an interpretation from Bah Lias research station. The Planter **78:**689-710.

78. **Flood, J., L. Keenan, S. Wayne, and Y. Hasan.** 2005. Studies on oil palm trunks as sources of infection in the field. Mycopathologia **159:**101-107.
79. **Fravel, D., C. Olivain, and C. Albouvette.** 2003. *Fusarium oxysporum* and its biocontrol. New Phytologist **157:**493-502.
80. **Fry, S. C.** 1988. The Growing Cell Wall: Chemical and Metabolic Analysis. Longman Scientific & Technical, Harlow.
81. **Fuchs, J.-G., Y. Moenne-Loccoz, and G. Defago.** 1997. Nonpathogenic *Fusarium oxysporum* strain Fo47 induces resistance to Fusarium wilt in tomato. Plant Disease **81:**492-496.
82. **Gao, S., G. H. Choi, L. Shain, and D. L. Nuss.** 1996. Cloning and targeted disruption of *enpg-1*, encoding the major *in vitro* extracellular endopolygalacturonase of the chestnut blight fungus, *Cryphonectria parasitica*. Applied and Environmental Microbiology **62:**1984-1990.
83. **Geiger, J. P., B. Rio, M. Nicole, and D. Nandris.** 1986. Biodegradation of *Hevea-Brasiliensis* Wood by *Rigidoporus-Lignosus* and *Phellinus-Noxius*. European Journal of Forest Pathology **16:**147-159.
84. **Gherbi, H., C. Delaruelle, M. A. Selosse, and F. Martin.** 1999. High genetic diversity in a population of the ectomycorrhizal basidiomycete *Laccaria amethystina* in a 150-year-old beech forest. Molecular Ecology **8:**2003-2013.
85. **Glen, M., I. C. Tommerup, N. L. Bougher, and P. A. O'Brien.** 2001. Interspecific and intraspecific variation of ectomycorrhizal fungi associated with *Eucalyptus* ecosystems as revealed by ribosomal DNA PCR-RFLP. Mycological Research **105:**843-858.
86. **Gold, M. H., and M. Alic.** 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Applied and Environmental Microbiology **58:**1832-1838.
87. **Gottlieb, A. M., E. Ferrer, and J. E. Wright.** 2000. rDNA analyses as an aid to the taxonomy of species of *Ganoderma*. Mycological Research **104:**1033-1045.
88. **Gronover, C. S., C. Schorn, and B. Tudzynski.** 2004. Identification of *Botrytis cinerea* genes up-regulated during infection and controlled by the G alpha subunit BCG1 using suppression subtractive hybridization (SSH). Molecular Plant-Microbe Interactions **17:**537-546.
89. **Hall, J. L., T. J. Flowers, and R. M. Roberts.** 1982. Cell Walls, Plant Cell Structure and Metabolism, 2nd ed. Longman, London.
90. **Hammel, K. E., B. Kalyanaraman, and T. K. Kirk.** 1986. Substrate free radicals are intermediates in ligninase catalysis. Proceedings of the National Academy of Science USA **83:**3708-3712.
91. **Hantula, J., M. Dusabenyagasani, and R. C. Hamelin.** 1996. Random amplified microsatellites (RAMS) - A novel method for characterizing genetic variation within fungi. European Journal of Forest Pathology **26:**159-166.
92. **Hantula, J., R. Kasanen, J. Kaitera, and S. Moricca.** 2002. Analyses of genetic variation suggest that pine rusts *Cronartium flaccidum* and *Peridermium pini* belong to the same species. Mycological Research **106:**203-209.

93. **Haran, S., H. Schickler, and I. Chet.** 1996. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. Microbiology **142:**2321-2331.
94. **Harborne, J. B., and C. A. Williams.** 2000. Advances in flavonoid research since 1992. Phytochemistry **55:**481-504.
95. **Harman, G. E., C. R. Howell, A. Viterbo, I. Chet, and M. Lorito.** 2004. *Trichoderma* species-opportunistic, avirulent plant symbionts. Nature Reviews Microbiology **2:**43-56.
96. **Harman, G. E., R. Petzoldt, A. Comis, and J. Chen.** 2004. Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and effects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. Phytopathology **94:**147-153.
97. **Hartley, C. W. S.** 1988. The Oil Palm (*Elaeis guineensis* jacq.), Third edition ed. Longman, UK.
98. **Hasan, Y., H. L. Foster, and J. Flood.** 2005. Investigations on the causes of upper stem rot (USR) on standing mature oil palms. Mycopathologia **159:**109-112.
99. **Hasan, Y., and P. D. Turner.** 1998. The comparative importance of different oil palm tissues as infection sources for basal stem rot in replantings. The Planter **74:**119-135.
100. **Hashim, K., T. K. Kim, and A. R. Abdul Razak.** 1991. Presented at the PORIM International Palm Oil Conference, 9-14 September.
101. **Henson, I. E.** 2003. Oil Palm-can it substitute the tropical rainforest? The Planter **79:**437-450.
102. **Hernandez-Perez, G., and J. L. Rols.** 1997. Degradation of lignosulfonated compounds by *Streptomyces viridosporus* strain T7A. Biotechnology Letters **19:**285-289.
103. **Highley, T. L., S. S. Barlev, T. K. Kirk, and M. J. Larsen.** 1983. Influence of O2 and Co2 on wood decay by heartrot and saprot Fungi. Phytopathology **73:**630-633.
104. **Hoegger, P. J., M. Navarro-Gonzalez, S. K. M. Hoffmann, and E. D. Westbrook.** 2004. The laccase gene family in *Coprinus cinerea* (*Coprinus cinereus*). Current Genetics **45:**9-18.
105. **Howell, C. R.** 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Disease **87:**4-10.
106. **Hseu, R. S., H. H. Wang, H. F. Wang, and J. M. Moncalvo.** 1996. Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. Applied and Environmental Microbiology **62:**1354-1363.
107. **Hunt, M. R. R., and C. A. Pilotti.** 2004. Low cost control for basal stem rot - a Poliamba initiative. The Planter **80:**173-176.
108. **Idris, A. S.** 1999. Basal stem rot (BSR) of oil palm (*Elaeis guineensis* jacq.) in Malaysia: factors associated with variation in disease severity. Ph.D. Wye College, University of London, London.
109. **Johansson, M., M. Denekamp, and F. O. Asiegbu.** 1999. Production and isozyme pattern of extracellular laccase in the S and P intersterility

groups of he root pathogen *Heterobasidion annosum*. Mycological Research **103:**365-371.
110. **Jourdan, C., and H. Rey.** 1997. Architecture and development of the oil palm (*Elaeis guineensis* jacq) root system. Plant and Soil **189:**33-48.
111. **Karlsson, M., A. Olson, and J. Stenlid.** 2003. Expressed sequences from the basidiomycetous tree pathogen *Heterobasidion annosum* during early infection of scots pine. Fungal Genetics and Biology **39:**51-59.
112. **Kersten, P. J., M. Tien, B. Kalyanaraman, and T. K. Kirk.** 1985. The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxy-benzenes. Journal of Biological Chemistry **260:**2609-2612.
113. **Kiiskinen, I. I., M. Ratto, and K. FKruus.** 2004. Screening for novel laccase producing-microbes. Journal of Applied Microbiology **97:**640-646.
114. **Kim, S., J. Song, and H. T. Choi.** 2004. Genetic transformation and mutant isolation in *Ganoderma lucidum* by restriction enzyme-mediated integration. FEMS Microbiology Letters **233:**201-204.
115. **Kirk, T. K.** 1987. Enzymatic combustion: The microbial degradation of lignin. Annual Review of Microbiology **41:**465-505.
116. **Kishore, G. K., S. Pande, J. N. Rao, and A. R. Podile.** 2005. *Pseudomonas aeruginosa* inhibits the plant cell wall degrading enzymes of *Sclerotium rolfsii* and reduces the severity of groundnut stem rot. European Journal of Plant Pathology **113:**315-320.
117. **Kortemaa, H., H. Rita, K. Haahtela, and A. Smolander.** 1994. Root-colonising ability of antagonistic *Streptomyces griseoviridis*. Plant and Soil **163:**77-83.
118. **Krauss, U., R. Bidwell, and J. Ince.** 1998. Isolation and preliminary evaluation of mycoparasites as biocontrol agents of crown rot of banana. Biological Control **13:**111-119.
119. **Krauss, U., and W. Soberanis.** 2001. Biocontrol of cocoa pod diseases with mycoparasite mixtures. Biological Control **22:**149-158.
120. **Kuster, E., and S. T. Williams.** 1964. Selection of media for isolation of *Streptomyces* spp. Nature **202:**928-929.
121. **Lascaris, D., and J. W. Deacon.** 1994. *In vitro* growth and microcycle conidiation of *Idriella bolleyi*, a biocontrol agent of cereal pathogens. Mycological Research **98:**1200-1206.
122. **Latiffah, Z., K. Harikrishna, S. G. Tan, S. H. Tan, F. Abdullah, and Y. W. Ho.** 2002. Restriction analysis and sequencing of the ITS regions and 5.8S Gene of rDNA of *Ganoderma* isolates from infected oil palm and coconut stumps in Malaysia. Annals of Applied Biology **141:**133-142.
123. **Lee, S. S.** 2000. The current status of root diseases of *Acacia magnium* Willd., p. 71-79. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
124. **Leonard, A. C.** 1998. Two *Ganoderma* species compared. Mycologist **12:**65-68.

125. **Lestan, D., M. Cernilec, A. Strancar, and A. Perdih.** 1993. Influence of surfactnants and related compounds on ligninolytic activity of *Phanerochaete chrysosporium*. FEMS Microbiology Letters **106:**17-22.
126. **Lev, S., and B. A. Horwitz.** 2003. A mitogen-activated protein kinase pathway modulates the expression of two cellulase genes in *Cochliobolus heterostrophus* during plant infection. Plant Cell **15:**835-844.
127. **Li, G., R. X. Li, Q. Y. Liu, Q. Wang, M. Chen, and B. J. Li.** 2006. A highly efficient polyethylene glycol-mediated transformation method for mushrooms. FEMS Microbiology Letters **256:**203-208.
128. **Likhitekaraj, S., and A. Tummakate.** 2000. Basal stem rot of oil palm in Thailand caused by *Ganoderma*, p. 69-70. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
129. **Lim, H. P., and Y. K. Fong.** 2005. Research on basal stem rot (BSR) of ornamental palms caused by basidiospores from *Ganoderma boninense*. Mycopathologia **159:**171-179.
130. **Lim, T. K., G. F. Chung, and W. H. Ko.** 1992. Basal Stem Rot of oil palm caused by *Ganoderma boninense*. Plant Pathology Bulletin **1:**147-152.
131. **Lorito, M., V. Farkas, S. Rebuffat, B. Bodo, and C. P. Kubicek.** 1996. Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. Journal of Applied Bacteriology **178:**6382-6385.
132. **Luard, E. J., and D. M. Griffin.** 1981. Effect of water potential on fungal growth and turgor. Transactions of the British Mycological Society **76:**33-40.
133. **Mahuku, G. S., and P. H. Goodwin.** 1998. Influence of sucrose, mucin and xanthan gum on spore germination of ten different fungi. European Journal of Plant Pathology **104:**849-852.
134. **Mahuku, G. S., and J. J. Riascos.** 2004. Virulence and molecular diversity within *Colletotrichum lindemuthianum* isolates from Andean and Mesoamerican bean varieties and regions. European Journal of Plant Pathology **110:**253-263.
135. **Martinez, A. T., M. J. Martinez, G. Almendros, M. Blanco, and A. E. Gonzalez.** 1994. Presented at the 5th International Mycological Congress, Vancouver, August 14-21.
136. **Martinez, D., L. F. Larrondo, N. Putman, M. D. S. Gelpke, K. Huang, J. Chapman, K. G. Helfenbein, P. Ramaiya, J. C. Detter, F. Larimer, P. M. Coutinho, B. Henrissat, R. Berka, D. Cullen, and D. Rokhsar.** 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nature Biotechnology **22:**695-700.
137. **Mayer, A. M., and R. C. Staples.** 2002. Laccase: new functions for an old enzyme. Phytochemistry **60:**551-565.
138. **Merrill, W.** 1992. Mechanisms to resistance of fungi in woody plants: a historical perspective, p. 1-12. *In* R. A. Blanchette and A. R. Biggs (ed.), Defence Mechanisms of Woody Plants Against Fungi. Spriner-Verlag, Berlin.

139. **Mikan-Venegas, J. F.** 2001. Arabinoxylan-degrading enzymes of the wheat pathogen. Ph.D. University of Bath, Bath.
140. **Miller, L.** 1994. Studies on CBH1: a cellobiohydrolase of *Sclerotinia sclerotium*. Ph.D. University of Sheffield.
141. **Miller, R. N. G.** 1995. The characterisation of *Ganoderma* in oil palm cropping systems. Ph.D. University of Reading.
142. **Miller, R. N. G., M. Holderness, and P. D. Bridge.** 2000. Molecular and morphological characterization of *Ganoderma* in oil-palm plantings, p. 159-182. *In* J. Flood, P. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Oxford.
143. **Miller, R. N. G., M. Holderness, P. D. Bridge, G. F. Chung, and M. H. Zakaria.** 1999. Genetic diversity of *Ganoderma* in oil palm plantings. Plant Pathology **48:**595-603.
144. **Miller, R. N. G., M. Holderness, P. D. Bridge, R. R. M. Paterson, M. Z. Hussin, and S. Meon.** 1995. Isozyme analysis for characterization of *Ganoderma* strains from South-East Asia. EPPO Bulletin **25:**81-87.
145. **Moncalvo, J. M.** 2000. Systematics of *Ganoderma*, p. 23-45. *In* J. Flood, P. Bridge, and M. Holderness (ed.), *Ganodema* Diseases of Perennial Crops. CABI Publishing, Wallingford.
146. **Moncalvo, J. M., H. F. Wang, and R. S. Hseu.** 1995. Gene phylogeny of the *Ganoderma lucidum* complex based on ribosomal DNA sequences. Comparison with traditional taxonomic characters. Mycological Research **99:**1489-1499.
147. **Moncalvo, J. M., H. H. Wang, and R. S. Hseu.** 1995. Phylogenetic-relationships in *Ganoderma* inferred from the internal transcribed spacers and 25s ribosomal DNA-sequences. Mycologia **87:**223-238.
148. **Morgan, F. J., and F. G. Priest.** 1981. Characterisation of a thermostable alpha-amylase from *Bacillus licheniformis* NCIB 6346. Journal of Applied Bacteriology **50:**107-114.
149. **Morrison, D. J.** 2004. Rhizomorph growth habit, saprophytic ability and virulence of 15 *Armillaria* species. Forest Pathology **34:**15-26.
150. **Morrison, D. J., and K. W. Pellow.** 2002. Variation in virulence among isolates of *Armillaria ostoyae*. Forest Pathology **32:**99-107.
151. **Mtui, G., and Y. Nakamura.** 2002. Continuous production of lignin-degrading enzymes by *Bjerkandra adusta* immobilized on polyurethane foam. Biotechnology Letters **24:**1743-1747.
152. **Mulligan, D. F. C., and J. W. Deacon.** 1992. Detection of presumptive mycoparasites in soil placed on host colonized agar plates. Mycological Research **96:**605-608.
153. **Nasir, N.** 2005. Diseases caused by *Ganoderma* spp. on perennial crops in Pakistan. Mycopathologia **159:**119-121.
154. **Navaratnam, S. J., and K. L. Chee.** 1965. Root inoculation of oil palm seedlings with *Ganoderma* sp. Plant Disease **49:**1011-1012.
155. **Nelson, N.** 1944. A photometric adaptation of the Somoygi method for the determinatin of glucose. Journal of Biological Chemistry:375-380.
156. **Nuss, D. L.** 1992. Biological control of chestnut blight: an example of virus-mediated attenuation of fungal pathogenesis. Microbiological Reviews **56:**561-576.

157. **Oeser, B., P. Heidrich, U. Muller, and P. Tudzynski.** 2002. Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea*/rye interaction. Fungal Genetics and Biology **36:**176-186.
158. **Olive, D. M., and P. Bean.** 1999. Principles and applications of methods for DNA-based typing of microbial organisms. Journal of Clinical Microbiology **37:**1661-1669.
159. **Onsando, J. M.** 1997. Distribution, severity and spread of *Armillaria* root diseases in Kenya tea plantations. Plant Disease **81:**133-137.
160. **Ooi, L. H., and Heriansyah.** 2005. Palm pulverisation in sustainable oil palm replanting. Plant Production Science **8:**345-348.
161. **Osbourn, A. E.** 1999. Antimicrobial phytoprotectants and fungal pathogens: A commentary. Fungal Genetics and Biology **26:**163-168.
162. **Panchal, G., and P. D. Bridge.** 2005. Following basal stem rot in young oil palm plantings. Mycopathologia **159:**123-127.
163. **Paterson, R. R. M., M. Holderness, J. Kelley, R. N. G. Miller, and E. B. O'Grady.** 2000. *In vitro* biodegradation of oil-palm stem using macroscopic fungi from South-East Asia: a preliminary investigation, p. 129-138. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
164. **Pearce, R. B.** 1996. Antimicrobial defences in the wood of living trees. New Phytologist **132:**203-233.
165. **Pearce, R. B.** 1990. Occurrence of Decay-Associated Xylem Suberization in a Range of Woody Species. European Journal of Forest Pathology **20:**275-289.
166. **Pearce, R. B.** 1991. Reaction Zone Relics and the Dynamics of Fungal Spread in the Xylem of Woody Angiosperms. Physiological and Molecular Plant Pathology **39:**41-55.
167. **Pearce, R. B., B. J. Fisher, T. A. Carpenter, and L. D. Hall.** 1997. Water distribution in fungal lesions in the wood of sycamore, *Acer pseudoplatanus*, determined gravimetrically and using nuclear magnetic resonance imaging. New Phytologist **135:**675-688.
168. **Penttila, M., and M. Saloheimo.** 1999. Lignocellulose breakdown and utilization by fungi, p. 272-293. *In* R. Oliver and M. Schweizer (ed.), Molecular fungal biology. Cambridge University Press.
169. **Penyalver, R., B. Vicedo, and M. M. Lopez.** 2000. Use of the genetically engineered *Agrobacterium* strain K1026 for the biological control of crown gall. European Journal of Plant Pathology **106:**801-810.
170. **Peries.** 1974. *Ganoderma* basal stem rot of coconut: a new record of the disease in Sri Lanka. Plant Disease Reporter **58:**293-295.
171. **Pilotti, C. A.** 2005. Stem rots of oil palm caused by *Ganoderma boninense*: Pathogen biology and epidemiology. Mycopathologia **159:**129-137.
172. **Pilotti, C. A., and P. D. Bridge.** 2002. Basal stem rot: probing the facts. The Planter **78:**365-370.
173. **Pilotti, C. A., F. R. Sanderson, and E. A. B. Aitken.** 2003. Genetic structure of a population of *Ganoderma boninense* on oil palm. Plant Pathology **52:**455-463.

174. **Pilotti, C. A., F. R. Sanderson, and E. A. B. Aitken.** 2002. Sexuality and interactions of monokaryotic and dikaryotic mycelia of *Ganoderma boninense*. Myological Research **11**:1315-1322.
175. **Pllotti, C. A., F. R. Sanderson, E. A. B. Aitken, and W. Armstrong.** 2004. Morphological variation and host range of two *Ganoderma* speices from Papua New Guinea. Mycopathologia **158**:251-265.
176. **Pilotti, C. A., F. R. Sanderson, E. A. B. Aitken, and P. D. Bridge.** 2000. Genetic variation in *Ganoderma* spp. from Papua New Guinea as revealed by molecular (PCR) methods, p. 195-204. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
177. **Prospero, S., O. Holdenrieder, and D. Rigling.** 2004. Comparison of the virulence of *Armillaria cepistipes* and *Armillaria ostoyae* on four Norway spruce provenances. Forest Pathology **34**:1-14.
178. **Qi, J. J., R. C. Ma, X. D. Chen, and J. Lan.** 2003. Analysis of genetic variation in *Ganoderma lucidum* after space flight. Advances in Space Research **31**:1617-1622.
179. **Raeder, U., W. Thompson, and P. Broda.** 1989. Genetic factors influencing lignin peroxidase activity in *Phenarochaete chrysosporium* ME 466. Molecular Microbiology **3**:919-924.
180. **Rao, V., C. C. Lim, C. C. Chia, and K. W. Teo.** 2003. Studies on *Ganoderma* spread and control. The Planter **79**:367-383.
181. **Rayner, A. D. M., and L. Boddy.** 1988. Fungal Decomposition of Wood: Its Biology and Ecology. John Wiley & Sons.
182. **Redfern, D. B., and J. Stenlid.** 1998. Spore dispersal and infection, p. 105-127. *In* S. Woodward, J. Stenlid, R. Karjalainen, and A. Huttermann (ed.), *Heterobasidion annosum*: Biology, Ecology and Control. CABI Publishing, Wallingford.
183. **Rehner, S. A., and G. J. Samuels.** 1994. Taxonomy and phylogeny of *Gliocladium* analysed by large ribosomal DNA sequences. Mycological Research **98**:625-634.
184. **Rishbeth, J.** 1963. Stump protection against *Fomes annosus*. III. inoculum with *Peniophora gigantea*. Annals of Applied Biology **52**:63-77.
185. **Rolph, H., R. Wijesekara, R. Lardner, F. Abdullah, P. M. Kirk, M. Holderness, P. D. Bridge, and J. Flood.** 2000. Molecular variation in *Ganoderma* isolates from oil palm, coconut and betelnut, p. 205-221. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
186. **Rotem, J., B. Wooding, and D. E. Aylor.** 1985. The role of solar radiation, especially ultraviolet, in the mortality of fungal spores. Phytopathology **75**:510-514.
187. **Ruzin, S. E.** 1999. Plant Microtechnique and Microscopy. Oxford University Press, New York.
188. **Ryvarden, L.** 1995. Can we trust morphology in *Ganoderma?*, p. 19-24. *In* P. K. Buchanan, R. S. Hseu, and J. M. Moncalvo (ed.), *Ganoderma*: Systematics, phytopathology and pharmacology. Proceedings of a contributed symposia 59A,B, Fifth International mycological congress. Taipei. Pp., Vancouver.
189. **Ryvarden, L.** 1991. Genera of Polypores. Fungiflora A/S, Oslo.

190. **Saha, B. C.** 2004. Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochemistry **39:**1871-1876.
191. **Samils, N., M. Elfstrand, D. L. L. Czederpiltz, J. Fahleson, A. Olson, C. Dixelius, and J. Stenlid.** 2006. Development of a rapid and simple *Agrobacterium tumefaciens*-mediated transformation system for the fungal pathogen *Heterobasidion annosum*. FEMS Microbiology Letters **255:**82-88.
192. **Samiyappan, R., R. Bhaskaran, and P. Rethinam.** 1996. Diagnosis for early detection of *Ganoderma diseases* in perennial crops: Approaches and prospects. Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection **103:**85-93.
193. **Sanderson, F. R.** 2005. An insight into spore dispersal of *Ganoderma boninense* on oil palm. Mycopathologia **159:**139-141.
194. **Sanderson, F. R., and C. A. Pilotti.** 1997. *Ganoderma* basal stem rot: an enigma, or just time to rethink an old problem? The Planter **73:**489-493.
195. **Sanderson, F. R., C. A. Pilotti, and P. Bridge.** 2000. Basidiospores: Their influence on our thinking regarding a control strategy for basal stem rot of oil palm, p. 113-119. *In* J. Flood, P. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing.
196. **Sankaran, K. V., P. D. Bridge, and C. Gokulapalan.** 2005. *Ganoderma* diseases of perennial crops in India - an overview. Mycopathologia **159:**143-152.
197. **Sariah, M., C. W. Choo, H. Zakaria, and M. S. Norihan.** 2005. Quantification and characterisation of *Trichoderma* spp. from different ecosystems. Mycopathologia **159:**113-117.
198. **Sariah, M., H. Joseph, and H. Zakariah.** 1997. Suppression of basal stem rot (BSR) of oil palm seedlings by calcium nitrate. The Planter **73:**359-361.
199. **Sariah, M., H. M.Z., M. R.N.G., and H. M.** 1994. Pathogenicity of *Ganoderma boninense* tested by inoculation of oil palm seedlings. Plant Pathology **43:**507-510.
200. **Sariah, M., and H. Zakaria.** 2000. The use of soil amendments for the control of basal stem rot of oil-palm seedlings, p. 89-100. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
201. **Savoie, J. M., G. Mata, and M. Mamoun.** 2001. Variability in brown line formation and extracellular laccase production during interaction between white-rot basidiomycetes and *Trichoderma harzianum* biotype Th2. Mycologia **93:**243-248.
202. **Scalet, M., E. Crivaletto, and F. Mallardi.** 1989. Demonstration of phenolic compounds in plant tissue by an osmium-iodide post fixation procedure. Stain technology **64:**273-290.
203. **Seubert, E.** 1997. Root anatomy of palms. Flora **192:**81-103.
204. **Shain, L.** 1967. Resistance of sapwood in stems of loblolly pine to infection by *Fomes annosus*. Phytopathology **61:**1143-1147.
205. **Shieh, M. T., R. L. Brown, M. P. Whitehead, J. W. Cary, P. J. Cotty, T. E. Cleveland, and R. A. Dean.** 1997. Molecular genetic evidence for

involvement of a specific polygalacturonase, P2c, in the invasion and spread in cotton bolls. Applied and Environmental Microbiology **63:**3548-3552.
206. **Shigo, A. L., and W. C. Shortle.** 1979. Compartmentalization of discolored wood in heartwood of red oak. Phytopathology **69:**710-711.
207. **Singh, G.** 1991. *Ganoderma*- the scourge of oil palm in the coastal areas. The Planter **67:**421-444.
208. **Singh, U. P., B. K. Sarma, and D. P. Singh.** 2003. Effect of plant growth-promoting rhizobacteria and culture filtrate of *Sclerotium rolfsii* on phenolic and salicylic acid contents in chickpea (*Cicer arietinum*). Current Microbiology **46:**131-140.
209. **Smith, B. J., and K. Sivasithamparam.** 2000. Internal transcribed spacer ribosomal DNA sequence of five species of *Ganoderma* from Australia. Mycological Research **104:**943-951.
210. **Smith, B. J., and K. Sivasithamparam.** 2000. Isozymes of *Ganoderma* species from Australia. Mycological Research **104:**952-961.
211. **Smith, B. J., and K. Sivasithamparam.** 2003. Morphological studies of *Ganoderma* (Ganodermataceae) from the Australasian and Pacific regions. Australian Systematic Botany **16:**487-503.
212. **Soylu, E. M., and S. Soylu.** 2003. Light and electron microscopy of the compatible interaction between *Arabidopsis* and the downy mildew pathogen *Peronospora parasitica*. Journal of Phytopathology **151:**300-306.
213. **Spratt, B. G.** 1999. Multilocus sequnce typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet. Current Opinion in Microbiology **2:**312-316.
214. **Stevenson, P. C., and M. P. Haware.** 1999. Maackiain in *Cicer bijugum* Rech. f. associated with resistance to *Botrytis* grey mould. Biochemical Systematics and Ecology **27:**761-767.
215. **Steyaert, R. L.** 1977. Basidiospores of two *Ganoderma* species and others of two related genera under the scanning electron microscope. Kew Bulletin **31:**437-442.
216. **Strange, R. N.** 2003. Inoculum Control, p. 121-142, Introduction to Plant Pathology. John Wiley & Sons, New York.
217. **Susanto, A., P. S. Sudharto, and R. Y. Purba.** 2005. Enhancing biological control of basal stem rot disease (*Ganoderma boninense*) in oil palm plantations. Mycopathologia **159:**153-157.
218. **Susanto, A., P. S. Sudharto, and Y. P. Rolletha.** 2002. Superior biocontrol agents of *Ganoderma boninense* pathogen of basal stem rot disease in the oil palm plantations. International Palm Oil Conference:1-6.
219. **Swedjemark, G., and J. Stenlid.** 2001. A highly diverse population of *Heterobasidion annosum* in a single stump of *Picea abies*. Mycological Research **105:**183-189.
220. **Tahvonen, R.** 1988. Microbial control of plant diseases with *Streptomyces* spp. EPPO Bulletin **18:**55-59.
221. **Talbot, N. J.** 2004. Plant-Pathogen Interactions. Blackwell Publishing, Oxford.

222. **Tapio, E., and A. Pohto-Lahdenpera.** 1991. Scanning electron microscopy of hyphal interaction between *Streptomyces griseoviridis* and some plant pathogenic fungi. Journal of Agricultural Science Finland **63:**435-441.
223. **Taylor, J. W., and M. C. Fisher.** 2003. Fungal multilocus sequence typing-it's not just for bacteria. Current Opinion in Microbiology **6:**351-356.
224. **Teeri, T.** 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends in Biotechnology **15:**160-167.
225. **Teeri, T., P. Lehtovaara, S. Kauppinen, I. Salovouri, and J. K. Knowles.** 1987. Homologous domains in *Trichoderma reesei* cellulolytic enzymes: gene sequence and expression of cellobiohydrolase II. Gene **51:**43-52.
226. **ten Hoopen, G. M., R. Rees, P. Aisa, T. Stirrup, and U. Krauss.** 2003. Population dynamics of epiphytic mycoparasites of the genera *Clonostachys* and *Fusarium* for the biocontrol of black pod (*Phytophthora palmivora*) and moniliasis (*Moniliophthora roreri*) on cocoa (*Theobroma cacao*). Mycological Research **107:**587-596.
227. **Thompson, A.** 1931. Stem-rot of the oil palm in Malaya. Bulletin Department of Agriculture, Straits Settlements and F.M.S., Science Series 6.
228. **Tien, M., and K. Kirk.** 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*:purification, characterisation, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proceedings of the National Academy of Science USA **81:**2280-2284.
229. **Tomashow, L. S., D. M. Weller, R. F. Bonsall, and L. S. P. Pierson III.** 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Applied and Environmental Microbiology **56:**908-912.
230. **Tuck, H. C., and K. Hashim.** 1997. Usefulness of soil mounding treatments in prolonging productivity of prime-aged *Ganoderma* infected palms. The Planter **73:**239-244.
231. **Tuohy, M. G., D. J. Walsh, P. G. Murray, M. Claeyssens, M. M. Cuffe, A. V. Savage, and M. P. Coughlan.** 2002. Kinetic parameters and mode of action of cellobiohydrolases produced by *Talaromyces emersonii.* Biochemica et Biophysica Acta **1596:**366-380.
232. **Turner, P. D.** 1981. Oil Palm Diseases and Disorders. Oxford University Press, Oxford.
233. **Utomo, C., and F. Niepold.** 2000. Development of diagnostic methods for detecting *Ganoderma*-infected oil palms. Journal of Phytopathology-Phytopathologische Zeitschrift **148:**507-514.
234. **Utomo, C., and F. Niepold.** 2000. The development of diagnostic tools for *Ganoderma* in oil palm, p. 235-248. *In* J. Flood, P. D. Bridge, and M. Holderness (ed.), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford.
235. **Utomo, C., S. Werner, F. Niepold, and H. B. Deising.** 2005. Identification of *Ganoderma*, the causal agent of basal stem rot disease in oil palm using a molecular method. Mycopathologia **159:**159-170.

236. **Vainio, E. J., and J. Hantula.** 2000. Genetic differentiation between European and North American populations of *Phlebiopsis gigantea.* Mycologia **92:**436-446.
237. **Vainio, E. J., K. Korhonen, and J. Hantula.** 1998. Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. Mycological Research **102:**187-192.
238. **Vainio, E. J., K. Lipponen, and J. Hantula.** 2001. Persistence of a biocontrol strain of *Phlebiopsis gigantea* in conifer stumps and its effects on within-species genetic diversity. Forest Pathology **31:**285-295.
239. **van der Vlugt-Bergmans, C. J. B., C. A. M. Wagemakers, D. C. T. Dees, and J. A. L. van Kan.** 1997. Cutinase A of *Botrytis cinerea* is expressed, but not essential during penetration of gerbera and tomato. Moecular Plant Microbe Interactions **10:**30-38.
240. **Varghese, G., and P. S. Chew.** 1973. *Ganoderma* root disease of lowland tea (*Camellia sinensis*) in Malaysia: some aspects of its biology and control. Malaysian Agricultural Research **2:**31-37.
241. **Vasiliauskas, R., and J. Stenlid.** 1999. Vegetative compatibility groups of *Amylostereum areolatum* and *A. chailletii* from Sweden and Lithuania. Mycological Research **103:**824-829.
242. **Venkatadri, R., and R. Irvine.** 1990. Effect of agitation on ligninase activty and ligninase production by *Phanerochaete chrysosporium.* Applied and Environmental Microbiology **56:**2684-2691.
243. **Verdera.** 2006. www.verdera.fi/homeeng.html.
244. **Viterbo, A., R. C. Staples, B. Yagen, and A. M. Mayer.** 1994. Selective mode of action of cucurbitacin in the inhibition of laccase formation in *Botrytis cinerea.* Phytochemistry **35:**1137-1142.
245. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. V. de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research **23:**4407-4414.
246. **Wakefield, E. M.** 1920. Diseases of the Oil Palm in West Africa. Kew Bulletin:306-308.
247. **Watanabe, T., Y. Ohnishi, Y. S. Kaizu, and T. Koshijima.** 1989. Binding site analysis of the ether linkages between lignin and hemicelluloses in lignin-carbohydrate complexes by DDQ-oxidation. Agricultural Biological Chemistry **53:**2233-2252.
248. **Weindling, R.** 1941. Experimental consideration of the mold toxin *Gliocladium* and *Trichoderma.* Phytopathology **31:**991-1003.
249. **Weller, D. M., J. M. Raaijmakers, B. B. McSpadden Gardener, and L. S. Thomashow.** 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annual Review of Phytopathology **40:**309-347.
250. **Whetton, R. W., J. J. MacKay, and R. R. Sedorff.** 1998. Recent advances in understanding lignin biosynthesis. Annual Review of Plant Physiology **49:**485-609.
251. **White, T. J., T. Bruns, S. Lee, and J. W. Taylor.** 1990. Amplification and direct sequencing of fungal RNA genes for phylogenetics., p. 315-322. *In* M. A. Innis, D. H. Gelgard, J. J. Sninsky, and T. J. White (ed.),

PCR Protocols: A Guide to Methods and Applications. Academic Press, New York.

252. **Williams, J., J. M. Clarkson, P. R. Mills, and R. M. Cooper.** 2003. Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus*. Applied and Environmental Microbiology **69:**4192-4199.

253. **Williams, J., J. M. Clarkson, P. R. Mills, and R. M. Cooper.** 2003. A selective medium for quantitative reisolation of *Trichoderma* from *Agaricus bisporus* compost. Applied and Environmental Microbiology **69:**4190-4191.

254. **Wood, T. M., A. Castanares, D. C. Smith, S. I. McCrae, and J. A. Brown.** 1992. Xylan-degrading enzyme systems of the fungi *Aspergillus awamori* and *Penicillium pinophilum*: studies on the isolation of over-producing mutant strains and aspects of the enzymology, p. 187-202. *In* J. Visser (ed.), Xylans and Xylanases. Elsevier Science Publishers.

255. **Woodward, S., J. Stenlid, R. Krajalainen, and A. Huttermann.** 1998. *Heterobasidion annosum*: Biology, Ecology Impact and Control. CABI Publishing, Wallingford.

256. **Yaropolov, A. I., O. V. Skorobogat'ko, S. S. Vartanov, and S. D. Varfolomeyev.** 1994. Laccase: properties, catalytic mechanism and applicability. Applied Biochemistry and Biotechnology **49:**257-279.

257. **Yedida, I., N. Benhamou, and I. Chet.** 1999. Induction of defence responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Applied and Environmental Microbiology **65:**1061-1070.

258. **Yee, D. C., D. Jahang, and T. K. Wood.** 1996. Enhanced expression and hydrogen peroxide dependence of lignin peroxidase from *Streptomyces viridosporus* T7A. Biotechnological Progress **12:**40-46.

259. **Young, J. C.** 1995. Microwave assisted extraction of the fungal metabolite ergosterol and total fatty acids. Journal of Agricultural Food Chemistry **43:**2904-2910.

260. **Zietkiewicz, E., A. Rafalski, and D. Labuda.** 1993. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics **20:**176-183.

261. **Zolan, M. E., and P. J. Pukkila.** 1986. Inheritance of DNA methylation in *Coprinus cinerus*. Molecular and Cellular Biology **6:**195-200.

Appendix

Isolate Number	Wood-Degrading Basidiomycetes
1	*Ganoderma boninense**
370923	*Ganoderma boninense**
BLRS 1	*Ganoderma boninense*
BLRS 2	*Ganoderma boninense*
SBJ 1	*Ganoderma boninense*
GMR3	*Ganoderma boninense*
GMB3	*Ganoderma boninense*
370902	*Lenzites* sp.*
370937	*Pycnoporus sanguinensis**
370939	*Hydnum* sp.*
370898	*Trametes hirsuta**
370942	*Marasmius* sp.*
370927	*Mycoacia* sp.*
2	*Corticium* sp.*
3	*Phlebiopsis gigantea#*
BLRS 9	*Coprinus* sp.
BLRS 7	Unidentified polyporous basidiomycete
SBJ 2	Unidentified

Table. 1a. Wood Degrading Basidiomycete Fungi. * Fungi were obtained from CAB International. # Fungus was obtained from the Forestry Commission UK. All other fungi were isolated from North Sumatra, Indonesia

Isolate Number	Deuteromycetes (anamorphic fungi), Imperfect Fungi
288054	*Gliocladium catenulatum**
300085	*Trichoderma virens **
288567	*Trichoderma virens **
APP 0023	*Clonostachys rosea**
APP 0043	*Clonostachys rosea**
AMR 0055	*Clonostachys byssicola**
AMR 0057	*Clonostachys byssicola**
APP 0129	*Trichoderma harzianum**
BLRS 3	*Trichoderma* sp.
BLRS 4	*Trichoderma* sp.
BLRS 5	*Trichoderma* sp.
BLRS 6	*Trichoderma* sp.
BLRS 10	*Clonostachys* sp.
BLRS 11	*Clonostachys* sp.
BLRS13	*Trichoderma* sp.
SBJ 9	*Trichoderma* sp.
SBJ 10	*Trichoderma* sp.

Table. 1b. Mycoparasitic/Antagonistic Deuteromycete Fungi. * Fungi were obtained from CAB International. All other fungi were isolated from windrows in North Sumatra.

Isolate Number	Morphology
SBJ 3	Orange spores, non-basidiomycete
SBJ 4	Orange spores, non-basidiomycete
SBJ 5	Orange spores, non-basidiomycete
SBJ 7	Orange spores, non-basidiomycete
BLRS 12	Black mycelium and spores, *Ceratocystis*-like

Table. 1c. Unidentified fungi isolated from a spore mass on windrows. All fungi were isolated from North Sumatra.

	Temperature estimation during the day (°C)				
Date	8am	10am	12am	2pm	4pm
6/5/04	26	26	27	28	29
7/5/04	25	27	27	29	29
8/5/04	25	26	27		
10/5/04	25	26	28	31	32
11/5/04	26	26	29	31	31
12/5/04	27	29	29	31	32
13/5/04	25	28	29	31	31
Average	25.6	26.9	28.0	30.2	30.7
St Dev	0.73	1.12	0.93	1.21	1.25

Table. 2a. Temperature from the soil of shaded palms.

	Temperature estimation during the day (°C)				
Date	8am	10am	12am	2pm	4pm
6/5/04	26	28	33	34	34
7/5/04	26	32	34	36	35
8/5/04	26	35	38		
10/5/04	27	38	44	42	38
11/5/04	26	36	40	40	38
12/5/04	27	39	42	42	40
13/5/04	25	39	40	39	37
Average	26.1	35.3	38.7	38.8	37.0
St Dev	0.64	3.77	3.73	2.97	2.00

Table. 2b. Temperature from the soil of non-shaded palms.

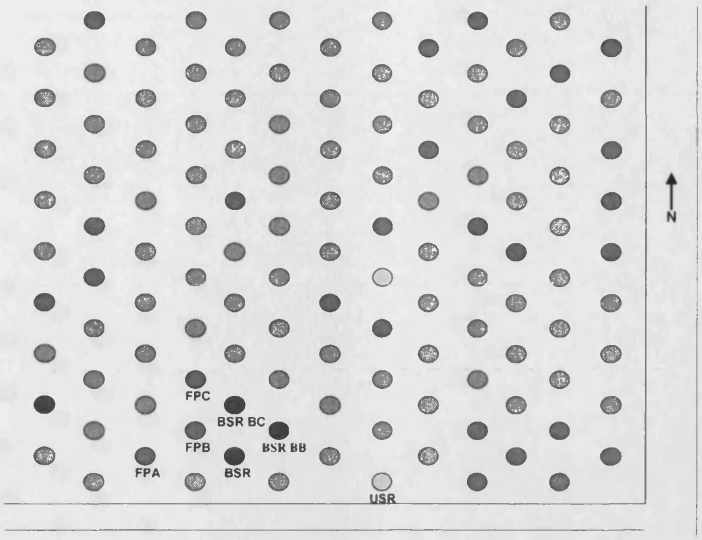

Fig. 1. Sampled palms from plot 86-400, Soneibijangkar estate.

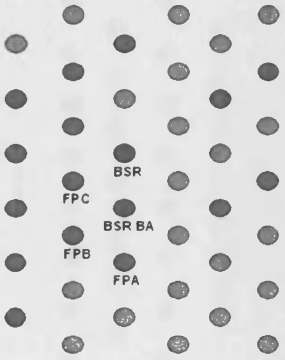

Fig. 2. Sampled palms from plot 84-300, **Soneibijangkar estate.** The USR palm is not shown as this palm was distant from BSR infected and fallen palms, from which samples were taken.

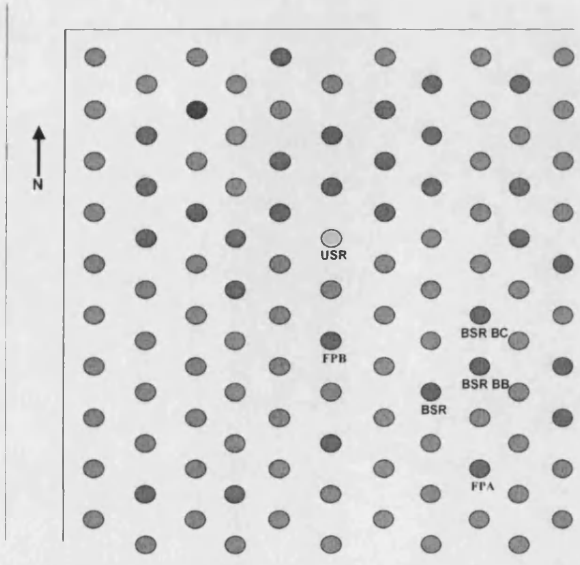

Fig. 3. Sampled palms from Plot 85-200, Bah Lias estate.

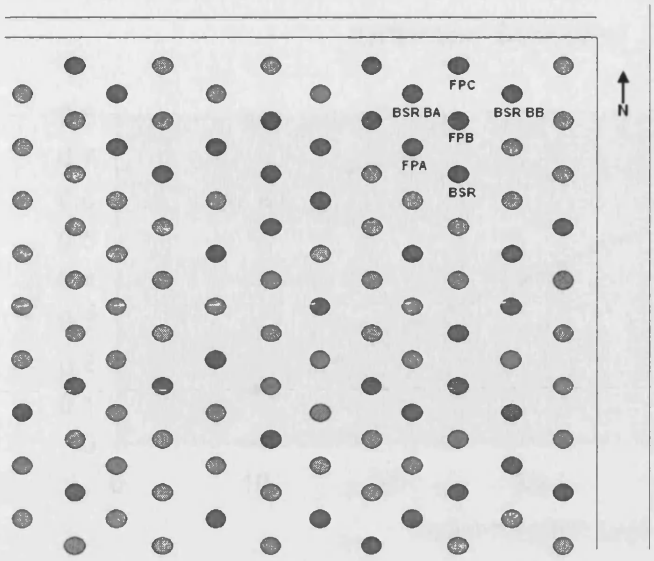

Fig. 4. Sampled palms from Plot 88-300, Bah Lias estate.

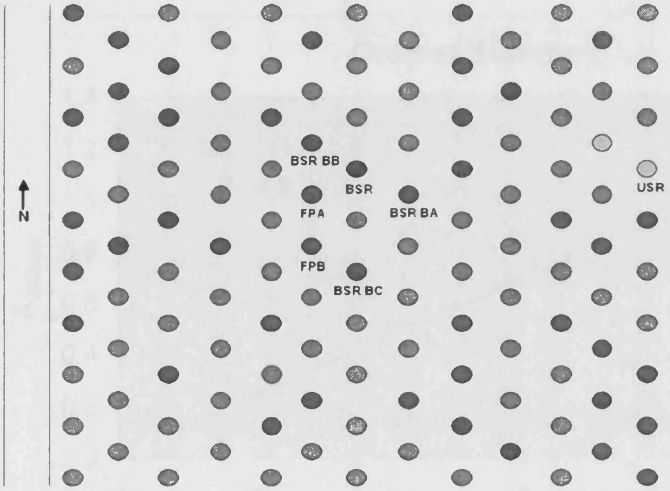

Fig. 5. Sampled palms from plot 86-200, Bah Lias estate.

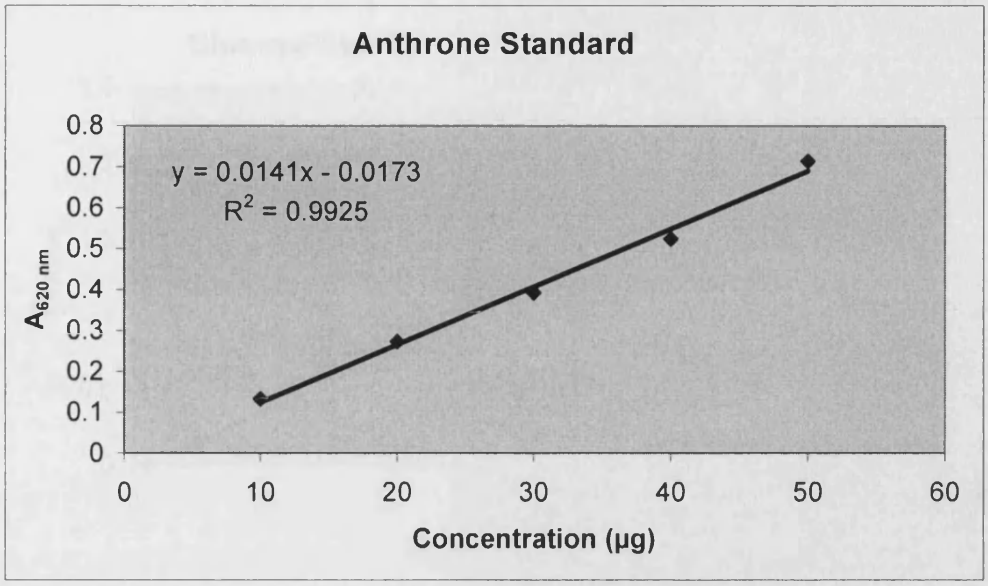

Fig. 6. Glucose concentration standard for quantification of hexose sugars using the anthrone assay.

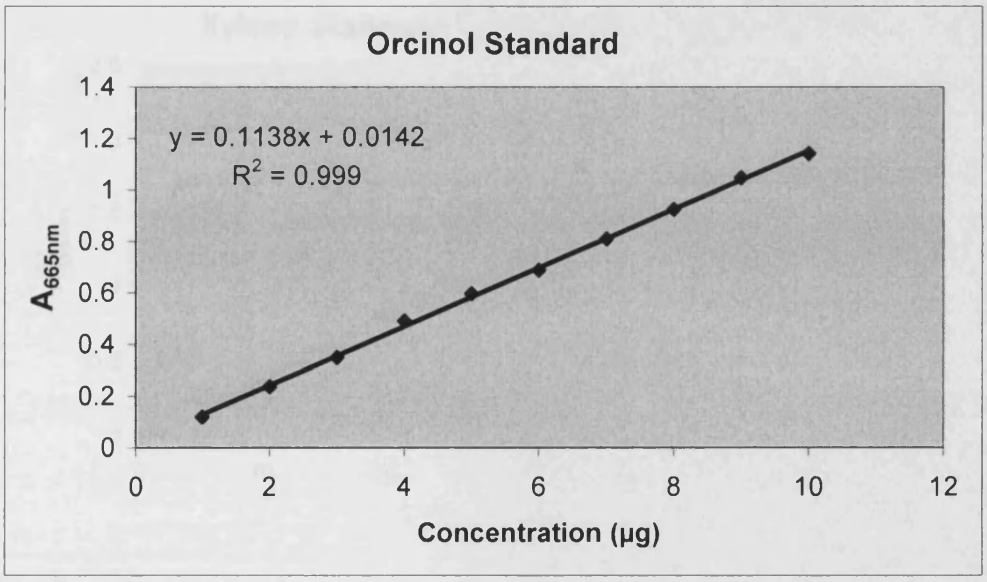

Fig. 7. Xylose concentration for quantification of pentose sugars using the orcinol assay.

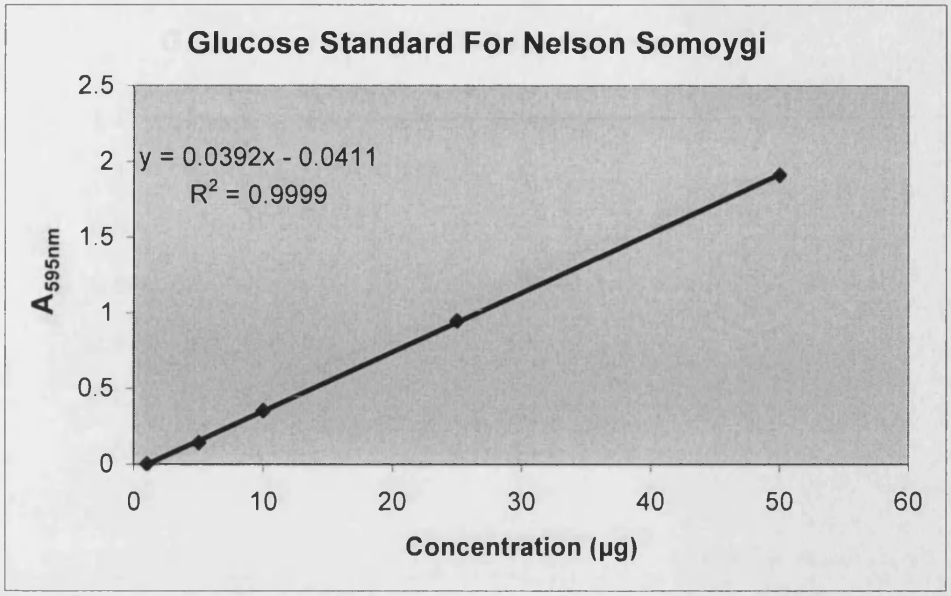

Fig. 8. Glucose standard for Nelson Somoygi reducing sugar assay.

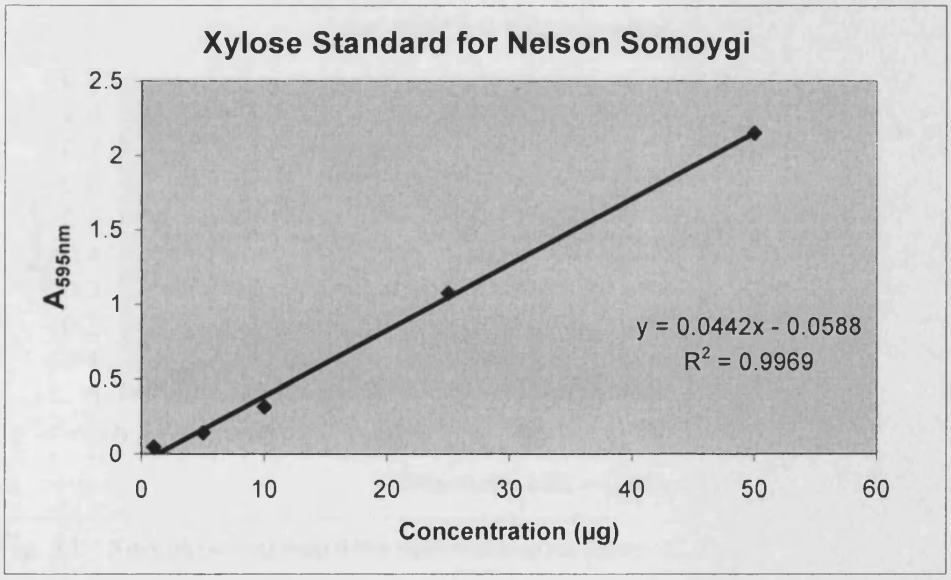

Fig. 9. Xylose standard for Nelson Somoygi reducing sugar assay.

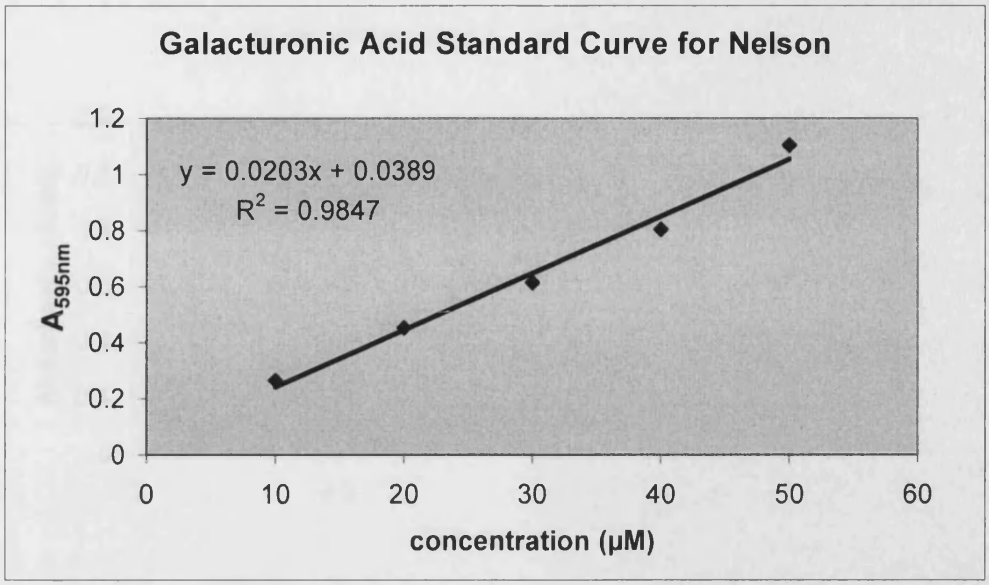

Fig. 10. Galacturonic acid standard for Nelson Somoygi reducing sugar assay.

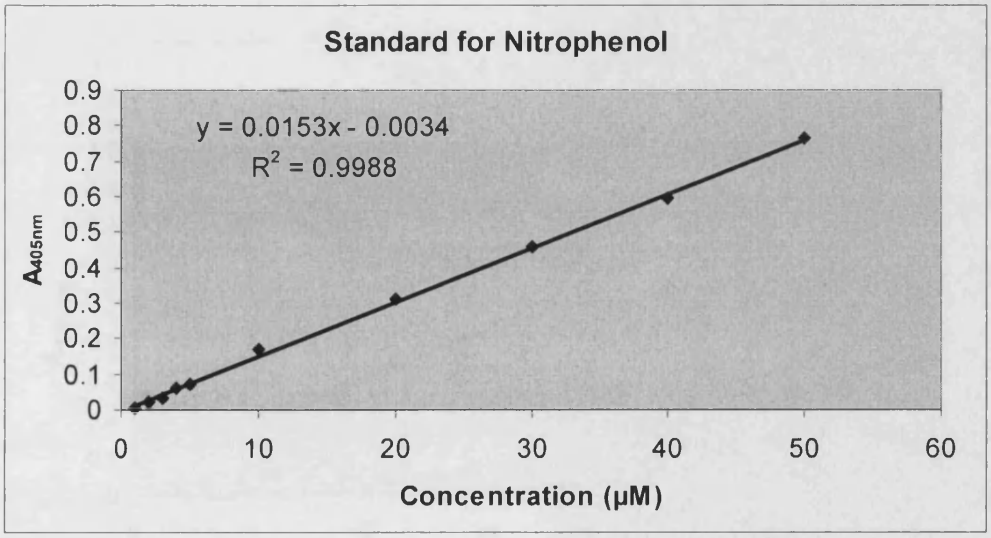

Fig. 11. Nitrophenol standard for galactosidase and glucosidase assays.

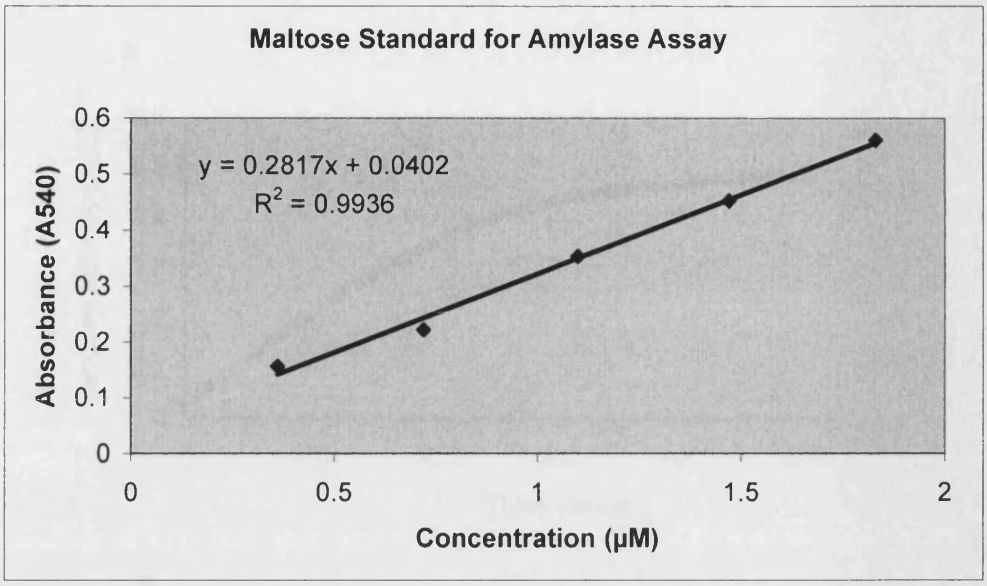

Fig. 12. Maltose standard for α-amylase reducing sugar assay.

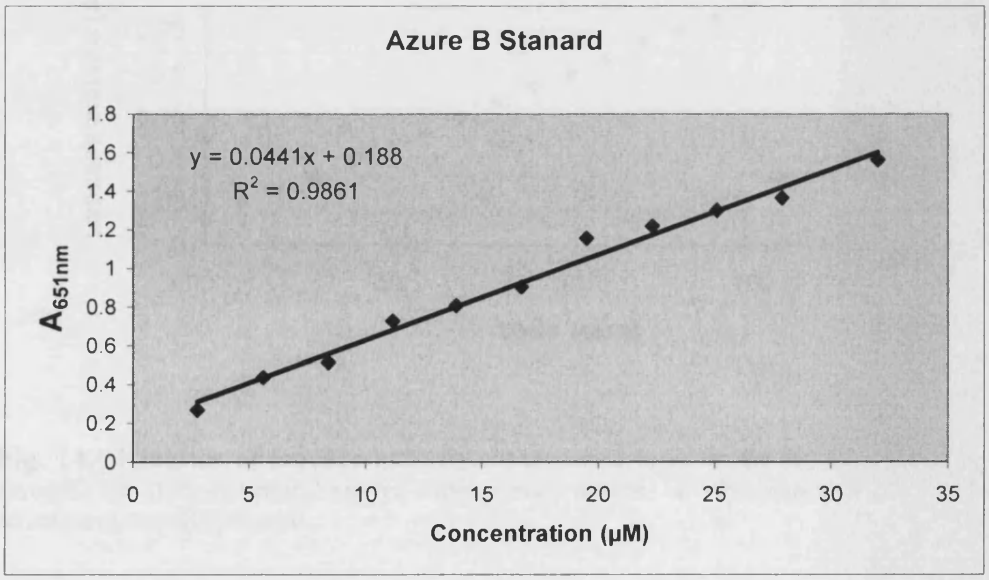

Fig. 13. Azure B standard for lignin peroxidase assay.

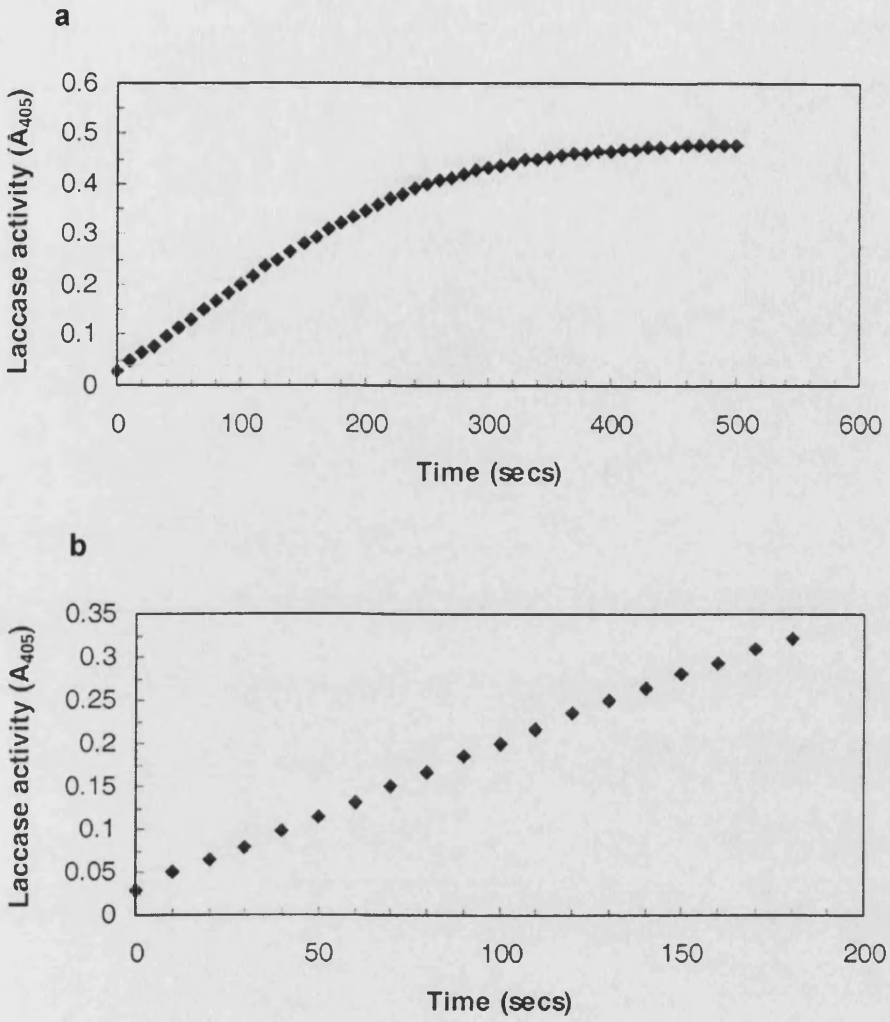

Fig. 14. Kinetics of laccase activity. Absorbance curve for the day 8 sample from the high nitrogen, 1% glucose, veratryl alcohol supplemented culture. **a.** Absorbance over 600 seconds. **b.** Absorbance over 200 seconds.

	Time (days)						
	2	*4*	*6*	*8*	*13*	*15*	*20*
High nitrogen, cell walls +							
Veratryl alcohol	0.006 ±0.002	0.055 ±0.016	0.009 ±0.007	0.016 ±0.016	0.054 ±0.000	0.020 ±0.018	0.052 ±0.008
Tryptophan	0.007 ±0.001	0.070 ±0.003	0.002 ±0.001	0.003 ±0.004	0.021 ±0.004	0.015 ±0.002	0.021 ±0.004
Syringic acid	0.000 ±0.000	0.000 ±0.000	0.023 ±0.032	0.060 ±0.082	0.000 ±0.000	0.000 ±0.000	0.266 ±0.025
High nitrogen, glucose +							
HN G VA	0.001 ±0.000	0.079 ±0.008	0.075 ±0.015	0.038 ±0.010	0.028 ±0.009	0.030 ±0.012	0.022 ±0.010
HN G Try	0.002 ±0.001	0.005 ±0.006	0.003 ±0.003	0.001 ±0.000	0.056 ±0.010	0.078 ±0.037	0.036 ±0.029
HN G SA	0.000 ±0.000	0.000 ±0.000	0.000 ±0.000	0.000 ±0.000	0.062 ±0.017	0.050 ±0.023	0.019 ±0.011
Low nitrogen, cell walls +							
Veratryl acohol	0.012 ±0.000	0.133 ±0.013	0.039 ±0.006	0.022 ±0.002	0.070 ±0.017	0.075 ±0.010	0.006 ±0.002
Tryptophan	0.006 ±0.000	0.069 ±0.001	0.013 ±0.005	0.022 ±0.022	0.107 ±0.052	0.058 ±0.004	0.097 ±0.038
Syringic acid	0.000 ±0.000	0.000 ±0.000	0.181 ±0.004	0.063 ±0.027	0.054 ±0.048	0.041 ±0.021	0.030 ±0.020
Low nitrogen, glucose							
Veratryl alcohol	0.014 ±0.001	0.171 ±0.106	0.074 ±0.021	0.082 ±0.079	0.189 ±0.115	0.124 ±0.127	0.062 ±0.062
Tryptophan	0.002 ±0.002	0.007 ±0.005	0.014 ±0.020	0.009 ±0.012	0.079 ±0.104	0.058 ±0.003	0.036 ±0.020
Syringic acid	0.000 ±0.000	0.007 ±0.006	0.001 ±0.001	0.011 ±0.014	0.094 ±0.088	0.023 ±0.033	0.035 ±0.007

Table. 3. Laccase activity from liquid cultures in low nitrogen/high nitrogen and glucose/cell wall carbon sources. Data cells show mean activity (AU/min) ± standard deviation of two samples.